PHENOLIC COMPOUNDS IN OIL-BEARING PLANTS AND THEIR INTERACTIONS WITH OILSEED PROTEIN ISOLATES

By

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Suggested Short Title: Phenolic Compounds in Oil-bearing Plants

DEDICATION

"To My Father and My Mother My Password for Success"

"To My Sisters, My Brothers and My Friends Source of My love"

ABSTRACT

Oil-bearing plants are important sources of edible oil and proteins; they have attracted attention recently because of their antioxidant, antimicrobial and anti-inflammatory properties. The objective of this work was to investigate the nature of phenolic compounds in oil-bearing plants and the effects of phenolic compounds on protein isolates from soybean and flaxseed. Proteins were isolated from full-fat and defatted soybean and flaxseed using sodium hydroxide extraction and isoelectric precipitation. Free phenolic compounds were extracted from the oil-bearing plants and protein isolates using methanol; for bound phenolic compounds the oil-bearing plants and proteins were subjected to basic and acidic hydrolysis followed by methanol extraction. Total free and bound phenolic compounds were determined by spectrophotometric analysis. Reversed phase-HPLC was used for separation of individual phenolic compounds, which were identified by mass spectrometry (MS). Molecular characteristics and biological properties of the protein isolates were studied using RP-HPLC, polyacrylamide gel electrophoresis (PAGE) and MS. Thermal and gelation properties of protein isolates were investigated using differential scanning calorimetry (DSC) and rheometry. Generally, bound phenolic compounds (20%-30% of total phenolic content) were higher in protein isolates from flaxseed than in protein isolates from soybean (10%-20% of total phenolic content). With flaxseed protein isolates, removal of phenolic compounds showed little effect on the electropherotic behavior of the proteins or the protein subunits. Native-PAGE, SDS-PAGE and RP-HPLC for the peptides profiles of hydrolyzed protein isolates from both full-fat and defatted soybean revealed the removal of free and bound phenolic compounds affect on the biological properties of protein isolates. Removal of free and bound phenolic compounds affected the thermal stability and gelation properties of protein isolates from flaxseed and soybean.

RÉSUMÉ

Les plantes oléagineuses sont d'importantes sources d'huiles comestibles et de protéines. Récemment, leurs propriétés anti-oxydantes, anti-microbiennes et anti-inflammatoires leur ont valu l'attention des chercheurs. Le présent travail visa une étude des composés phénoliques des plantes oléagineuses, et l'effet de ceux-ci sur des protéines isolées à partir de graines soya (*Glycine max* (L.) Merr.) et de lin (*Linum usitatissimum* L.). Les protéines furent isolés à partit de soja non-dégraissé, de soya dégraissé et de graines de lin, en employant une extraction à l'hydroxyde de sodium suivi d'une précipitation isoélectrique. Les composés phénoliques libres des plantes oléagineuses et des protéines isolés furent extraits au méthanol, tandis que pour les composés phénoliques liés, le matériel subit une hydrolyse alcaline puis une hydrolyse acide avant l'extraction au méthanol. La somme des composés phénoliques libres et liés fut déterminé par analyse spectrophotométrique. Les composés phénoliques individuels furent séparés par chromatographie à polarité de phase inversée (PI-HPLC), et indentifiés par spectrométrie de masse (SM). Les caractéristiques moléculaires et biologiques des protéines furent étudiés par RP-HPLC, électrophorèse en gel de polyacrylamide (EGPA) et par SM. Les propriétés thermiques et de gélification des protéines furent étudiés par analyse calorimétrique à compensation de puissance et par rhéomètre. En général, les composés phénoliques liés, représentant de 20% à 30% du total des composés phénoliques présents dans les protéines isolés du lin, furent plus élevés que ceux provenant des protéines isolées du soya, où ils ne représentèrent que 10% à 20% du total des composés phénoliques. Pour les protéines du lin, d'enlever les composés phénoliques n'eut que très peu d'effet sur le comportement électrophorétique de celles-ci ou de leurs protomères. Les profils d'EGPA sous conformation native, ou avec SDS, ainsi que de PI-HPLC des peptides provenant de soja non-dégraissé, de

même que de soya dégraissé démontrèrent que d'enlever les composés phénoliques libres et liés eut un effet significatif sur les propriétés biologiques des protéines isolées. De plus, d'enlever les composés phénoliques libres et liés eut un effet significatif sur la stabilité thermique et la gélification des protéines isolées du lin et du soya.

PREFACE

CLAIMS TO ORIGINAL RESEARCH

The original contributions for this work are as following:

1. This represents the first study on the distribution of phenolic compounds as free and bound phenolic compounds in both full-fat and defatted soybean and flaxseed as well as the protein isolates from flaxseed and soybean.

2. This is first comprehensive study to investigate the effects of removal of free and bound phenolic compounds on the antioxidant activity, molecular characteristics, proteolysis and physico-chemical properties of protein isolates from full-fat and defatted soybean and flaxseed.

ACKNOWLEDGEMENTS

In the Name of God, the Most Gracious and the Most Merciful

''Verily, my prayer, my sacrifice, my living, and my dying are for God, the lord of mankind''

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LIST OF ABBREVIATIONS

AA	**Antioxidant Activity**
ABS	**Absorbance**
BSA	**Bovine Serum Albumin**
Da	**Dalton**
DfI	**Protein Isolate from Defatted Flaxseed**
DPPH	**2, 2-Diphenyl Picryl Hydrazyl**
DSC	**Differential Scanning Calorimetry**
DsI	**Protein Isolate from Defatted Soybean**
ESI-MS	**Electrospray Ionization-Mass Spectrometry**
FfI	**Protein Isolate from Full-fat Flaxseed**
FsI	**Protein Isolate from Full-fat Soybean**
G'	**Storage Moduli**
G"	**Loss Moduli**
HDL	**High Density Lipoprotein**
KDa	**Kilo Dalton**
LDL	**Low Density Lipoprotein**
m/z	**Mass to Charge Ratio**
MS	**Mass Spectrometry**
MW	**Molecular Weight**
PAGE	**Polyacrylamide Gel Electrophoresis**
RP-HPLC	**Reversed Phase High Performance Liquid Chromatography**
RM	**Relative Migration**
RT	**Retention Time**

SECO	**Secoisolariciresinol**
SDG	**Secoisolariciresinol Diglucoside**
SDS	**Sodium Dodecyl Sulfate**
TBA	**Thiobarbituric Acid**
WHC	**Water Holding Capacity**

CHAPTER 1

INTRODUCTION

1.1 General

Traditionally, oil-bearing plants have been used as important sources of oil and proteins. Examples include soybean, canola, flaxseed and olive. Recently, phenolic compounds in these oil bearing plants have become components of interest due to their therapeutic properties such as anti-cancer, anti-viral, anti-inflammatory, hypolipidimic, and hypoglycemic. Recently, the nutritional interest in phenolic compounds has been due impart to deleterious effects that are caused by the ability of certain phenolic compounds to bind with macromolecules such as proteins, lipid and carbohydrates (Bravo, 1998). The types, amounts and properties of phenolic compounds in these foods vary tremendously (Lipworth *et al.*, 1997; Bravo, 1998; Escarpa and Gonzalez, 2001; Toschi *et al.*, 2000). Two classes of phenolic compounds have been identified in oil-bearing plants; simple phenolic compounds (How and Morr, 1982) and complex phenolic compounds (Johnsson *et al.*, 2000).

There is relatively little information on the interactions of phenolic compounds in plants the most other food components, e.g. proteins, carbohydrates and lipids, although they are known to occur frequently (Bravo *et al.*, 1992). The interactions of phenolic compounds with other food components in complex food systems affect the palatability of foods (Beauchamp and Maller, 1977). Interactions of tannins with other food components such as protein or polysaccharide components are known to occur (Bravo, 1998). The chemical nature of phenolic compounds allow them to interact with other food components such as proteins due to the presence of hydrogen bonding (Loomis and Battaile, 1966), covalent bonding (Mason, 1955), hydrophobic interactions (Hagerman and Butler, 1978) and ionic bonding (Rubino *et al.*, 1996). The mechanisms of the interaction between the phenolic compounds and the major food components are not well established.

The association of phenolic compounds with proteins may affect functional properties (eg; gelling properties, emulsification, and water holding capacity) and biological properties of the protein. Most commonly, the association of the phenolic compounds with carbohydrates and lipids impart antioxidant properties to these major food components (Ratty and Das, 1988; Sabally, 2006). In some cases, the functional properties of edible oil are influenced by the interaction of lipid components with phenolic compounds (Bendini *et al.*, 2001; Pelillo *et al.*, 2002). These protein-phenolic interactions and lipid-phenolic interactions have been studied both in vitro and vivo (Quesada *et al.*, 1996, Longstaff *et al.*, 1993). This research will address identification of phenolic compounds in oil-bearing plants and the effect of protein-phenolic interactions on molecular, biological and functional properties of food proteins.

1.2 Objectives

The overall objective of this study was to investigate phenolic compounds present in oil-bearing plants and the nature of phenolic interactions with soybean and flaxseed proteins.

The specific objectives of the study were:

1). To determine the contents of free and bound phenolic compounds in oil-bearing plants and to identify the major phenolic compounds and their antioxidant activity in soybean, flaxseed and olive using reversed phase high performance liquid chromatography (RP-HPLC) and electrospray ionization mass spectrometry (ESI-MS) (Chapter 3).

2). To investigate phenolic interactions with protein isolated from soybean and flaxseed by studying the effects of removal of phenolic compounds on molecular characteristics of the isolated proteins (Chapter 4).

3). To investigate the effects of removal of phenolic compounds on in *vitro* proteolysis of protein isolates from soybean and flaxseed using the human digestive enzymes (Chapter 5).

4). To investigate the effects of removal of phenolic compounds on physico-chemical properties of soybean and flaxseed protein isolates by measuring denaturation temperature, water holding capacity (WHC) and rheological properties (Chapter 6).

CHAPTER 2

LITERATURE REVIEW

2.1 Properties, Occurrence and Types of Phenolic and Polyphenolic Compounds in Oil-Bearing Plants

2.1.1 Phenolic Compounds

Phenolic compounds are divided according to the chemical structures into simple phenols (such as phenol, cresol, thymol and orcinol), phenolic acids (such as gallic, protocatechuic, vanillic and syringic acids), aldehyde forms of phenolic acids (e.g. vanillin, syringaldehyde and *p*-hydroxybenzaldehyde), phenylacetic acids, acetophenones, phenylpropanoid and their derivatives, chromones and coumarins (e.g. umbilliferone and aesculetin), and cinnamyl alcohols (such as coniferyl, sinapyl, syringyl and *p*-coumaryl alcohols). Phenolic compounds exist in food in both free form and bound to other food components due the chemical structures and natures (Bravo, 1998). Four forms of phenolic compounds have been reported in oil-seeds including free, esterified, etherified and in-soluble bound phenolic acids (Kozlowska *et al.*, 1975; Krygier *et al.*, 1982; Kozlowska *et al.*, 1983; Dabrowski and Sosulski, 1984; Naczk and Shahidi, 1989).

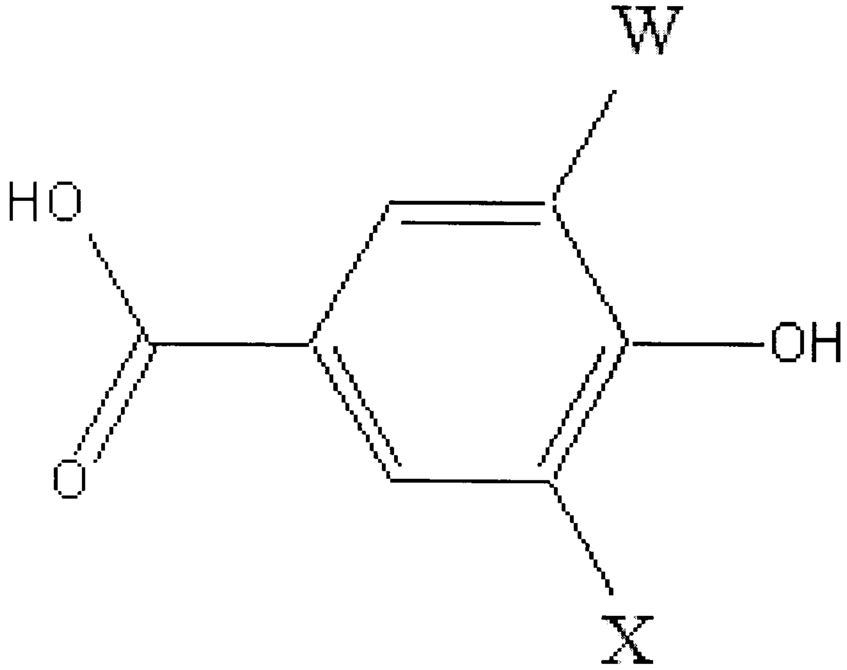
Phenolic acids have nomenclatures that depend on their chemical composition and structural. Shahidi and Naczk (2004) reported that phenylpropanoid and cinnamic acid derivatives are called phenolic acids. Figures 2.1A and 2.1B show the chemical structure of phenolic acids identified in oil-bearing plants. Flaxseed contains phenylpropanoids such as *p*-coumaric, *o*-coumaric, ferulic, *p*-hydroxybenzoic, vanillic and sinapic acids in the free form, while *p*-coumaric acid glycoside, ferulic acid glucoside and *p*-coumaric glucoside exist in bound forms (Klosterman *et al.*, 1955; Kozlowska *et al.*, 1983; Westcott and Muir, 1996; Johnsson *et al.*, 2000). Dabrowski and Sosulski (1984) reported that the content of esterified, free and bound phenolic compounds were 81 mg, 73.9 mg and 7.2 mg/100 g, respectively, in dehulled defatted flaxseed meal. Varga and Diosady (1994) found total phenolic content between 0.355-0.442 g/100 g in flaxseed meals, while insoluble bound phenolic compounds comprised 26%-29% of the total phenolic compounds. Oomah *et al.* (1995) reported a total phenolic content of 8-10 g/kg in flaxseed; the esterified phenolic acids covered 48%-66% of total phenolic compounds. Phenolic compounds that are not released after extraction and alkaline hydrolysis were assumed to be ether bound phenolic compounds (Oomah *et al.*, 1995). Tables 2.1, 2.2 and 2.3 show the distribution of total, esterified, free and bound phenolic acids in flaxseed.

The content of free phenolic compounds in soybean flake was 25.6 mg/100 g (Maga and Lorenz, 1974), while Dabrowski and Sosulski (1984) showed that soybean flour contains 73.6 mg/100 g phenolic compounds. Kozlowska *et al.* (1991) reported that the content of phenolic compounds was 0.23 mg/g (dry weight basis) in soybean flour; Naczk *et al.* (1986) reported that the soybean meal contains 4.6 mg/g phenolic compounds. Ethanol extraction of defatted soy flour revealed that the syringic acid is the predominant phenolic acid (Arai *et al.*, 1966). The major phenolic compounds identified in soybean flour are ferulic, syringic and vanillic acids (Maga and Lorenz, 1974). Dabrowski and Sosulski (1984) reported that syringic acid is the predominant free phenolic acid in soybean flour. While How and Morr (1982) demonstrated that *o*-coumaric and *p*-coumaric and ferulic acid were major phenolic acids. Mega and Lorenz (1974) found a wide range for distribution of free phenolic acids in soybeans, peanuts and cottonseeds flours with syringic, ferulic and vanillic acids being the major components. Tables 2.1, 2.2 and 2.3 show the distribution of phenolic compounds in soybean.

Sunflower is considered an important source of oil with considerable amounts of certain phenolic constituents. Chlorogenic acid had been reported as the major phenolic acid in sunflower kernels with much lower content of caffeic acid (Milic *et al.*, 1968; Mikolajczak *et al.*, 1970). Leung *et al.* (1981) found that the predominant bound phenolic acids among seven cultivars of sunflowers is caffeic acid. Tables 2.1, 2.2 and 2.3 show the free and bound phenolic compounds in sunflower seeds.

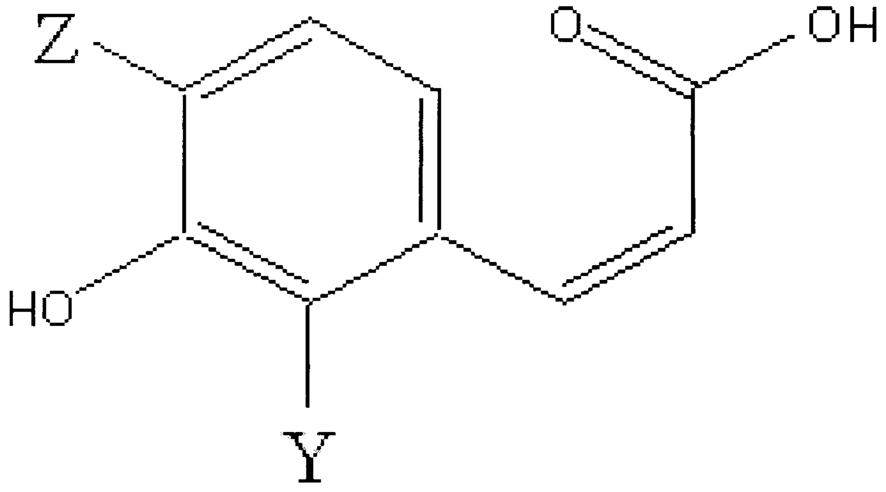
Full-fat rapeseed, flour contains phenolic compounds 10%-30% higher than O*leaginous* seeds. The phenolic compounds content in defatted rapeseed meal is higher than defatted soybean meal (Malcolmson *et al.*, 1978; Sosulski, 1979; Ismail *et al.*, 1981; Shahidi and Naczk, 1992). The phenolic compounds in rapeseed have been classified according to occurrences to free, esterified, and insoluble-bound form that are derived from benzoic and cinnamic acids. The total content of phenolic compounds in defatted rapeseed meals is 18.4 g/kg, while full-fat rapeseed flour contains 6.2-12.8 g/kg (Naczk *et al.*, 1992). Sinapine, the major phenolic compounds of rapeseed has been found to vary from 1.2%-2.5% for defatted meal (Clandinin, 1961; Austin and Wolff, 1968) depending on location and cultivars (Mueller *et al.*, 1978). Similarly, wide range of sinapine values (0.4%-1.8%) on dry matter basis was reported for several cultivars of *Cruciferae* crops including the *Brassica* species (Kerber and Buchloh, 1980). Kozlowska *et al.* (1991) reported that the rapeseed contains phenolic compounds such as *p*-hydroxybenzoic acid, vanillic acid, genestic, protocatechuic acid, syringic acid, *p*-coumaric acid, cis-and trans-ferulic acid, caffeic acid and chlorogenic acid in the free form. Krygier *et al.* (1982) reported that the predominant phenolic acids are esterified phenolic acids present in rapeseed protein products, and contribute to 80% of the total phenolic compounds. Full-fat flours of rapeseed contain higher contents of esterified phenolic acids compared to defatted meal (Krygier *et al.*, 1982); this author reported that the predominant phenolic acid in both rapeseed meal and flour is sinapic acid, which contributes 71%-97% of the total phenolic acid liberated from esterified phenolic acids. Pokorny and Reblova (1995) reported that sinapine; the choline ester of sinapic acid is the most predominant phenolic choline ester present in rapeseed flour and meal. Blair and Reichert (1984) reported that the content of sinapine in defatted rapeseed and canola are 2.67%-2.85%. Durkee and Thivierge (1975) and Kozlowska *et al.* (1983) identified sinapic acid as the most insoluble, bound phenolic compound in rapeseed flour; sinapic acid contributed 30%-59% of the total insoluble fraction of phenolic compounds. The most abundant glycoside sinapate in rapeseed/canola was 1-O-β-D glucopyranosyl sinapate (Amarowicz and Shahidi, 1994; Amarowicz *et al.*, 1995). Naczk and Shahidi (1989) reported the content of insoluble bound phenolic compounds in canola meal is 1 g/kg. Kozlowska *et al.* (1983) found that the content of insoluble bound phenolic compounds in rapeseed/canola flour is 32-50 mg/kg. Tables 2.1, 2.2 and 2.3 show the distribution of phenolic compounds in rapeseed.

Simple phenolic compounds identified in olive meals included tyrosol, hydroxytyrosol, gallic, protocatechuic, *p*-hydroxybenzoic, vanillic, caffeic, syringic, *p*-coumaric and *o*-coumaric, ferulic and cinnamic acids (Montedoro *et al.*, 1992; Akasbi *et al.*, 1993; Tsimidou, 1998).



Phenolic Acids	W	X
Protocatechuic	H	OH
Vanillic	OCH_3	H
Syringic	OCH_3	OCH_3
Gallic	OH	OH
p-Hydroxybenzoic	H	H

Figure 2.1A: Structures of some phenolic acids found in oil-bearing plants (Shahidi and Naczk, 2004).



Phenolic Acids	Y	Z
p-Coumaric	H	H
Caffeic	H	OH
Ferulic	H	OCH_3
Sinapic	OCH_3	OCH_3

Figure 2.1B: Structures of phenolic acids found in oil-bearing plants (Shahidi and Naczk, 2004).

Table 2.1: Distribution of phenolic compounds in different oil-bearing plants for different cultivars.

	Phenolic Compounds (g/kg)			
Oil-bearing Plant	**Total**	**Free**	**Esterified**	**Insoluble Bound**
Flaxseed[a]	7.46-10.55	NR	4.79-5.42	2.67-5.13
Soybean[b]	1.43-2.25	NR	NR	NR
Rapeseed[c]	6.30-18.87	0.60-2.62	5.70-15.20	0.00-1.05
Olive[d]	0.82-1.71	NR	NR	NR

NR: Not Reported.
Sources:
(a): Prairie Registration Recommending Committee for Grain (1994); Oomah and Mazza (1998).
(b): Genovese *et al.* (2005).
(c): Krygier *et al.* (1982); Kozlowska *et al.* (1983); Kozlowska *et al.* (1975); Naczk and Shahidi (1989).
(d): Boskou *et al.* (2006).

Table 2.2: Phenolic acids liberated from soluble esters of oil-seed flours (mg/100 g).

Phenolic Acid	Soybean	Flaxseed	Rapeseed	Sunflower
p-Hydroxybenzoic acid	13.00	2.60	5.60	6.00
Vanillic acid	0.00	Trace	0.70	0.80
Protocatechuic acid	0.00	0.00	Trace	0.00
Syringic acid	26.40	0.40	1.10	2.20
Trans-*p*-Coumaric acid	9.40	4.90	Trace	5.60
Trans-Ferulic acid	14.50	33.30	15.00	5.80
Trans-Caffeic acid	5.20	3.60	Trace	960.90
Cis-Sinapic acid	0.00	0.00	33.00	0.00
Trans-Sinapic acid	0.00	29.10	1110.70	0.00
Total	68.50	73.90	1166.10	981.30

Source: Dabrowski and Sosulski (1984).

Table 2.3: Phenolic acids librated from insoluble residue of oil-seed flours (mg/100 g).

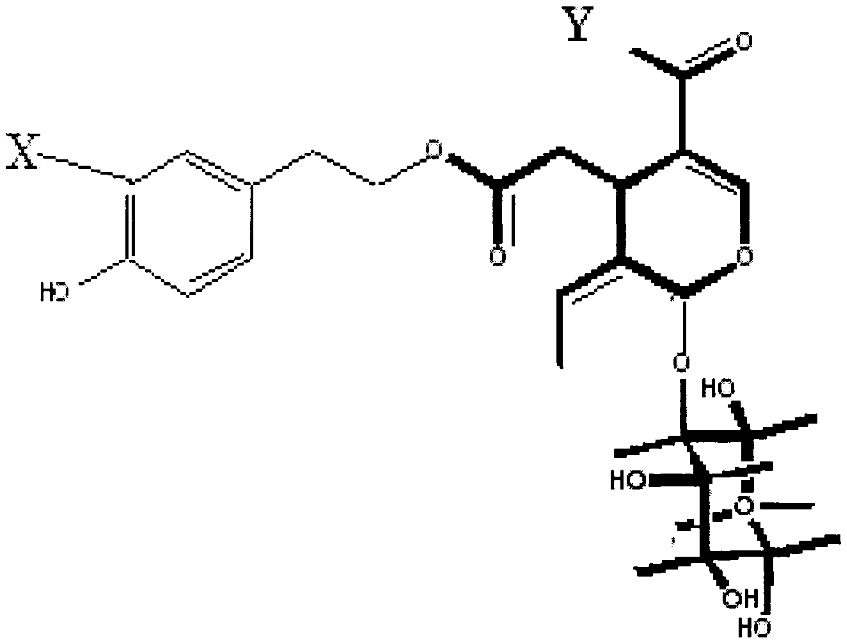
Phenolic Acid	Soybean	Flaxseed	Rapeseed	Sunflower
p-Hydroxybenzoic acid	0.90	Trace	0.00	1.40
Syringic acid	2.20	0.00	0.00	1.40
Trans-*p*-Coumaric acid	0.00	1.20	0.00	Trace
Trans-Ferulic acid	1.20	4.30	0.00	1.40
Trans-Caffeic acid	0.80	1.70	0.00	18.20
Total	5.10	7.20	0.00	22.40

Source: Dabrowski and Sosulski (1984).

Simple phenolic acids have ability to associate strongly with primary metabolites such as protein and carbohydrate (Vekey *et al.*, 1997). Table 2.1 shows the distribution of phenolic compounds in olive meal. The major phenolic acids identified in olive include hydroxycinnamic acid, hydroxybenzoic acid, hydroxycaffeic acid and hydroxyphenylacetic acid (Servili *et al.*, 1999; Bianco and Uccella, 2000). Cinnamic acid and 2-4 (-hydroxyphenyl) ethyl acetate have been detected in olive oil (Brenes-Balbuena *et al.*, 1999).

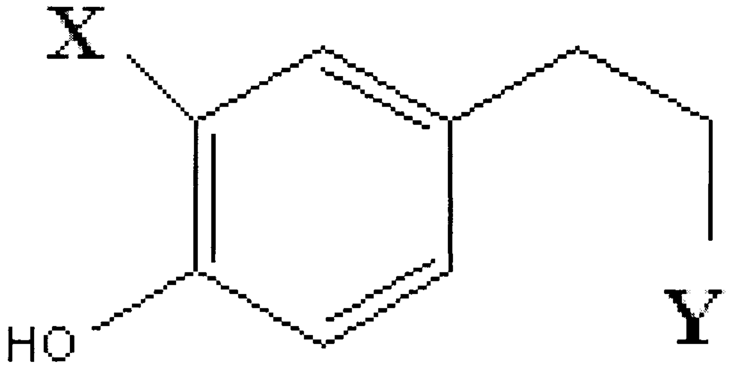
Other phenolic compounds in olive include aglycons which are derived from oleuropein and ligstroside (Montedoro *et al.*, 1992; Montedoro *et al.*, 1993; Angerosa *et al.*, 1995; Zunin *et al.*, 1995). Oleuropein is an ester of 2'-(3', 4'-dihydroxyphenyl) ethanol (hydroxytyrosol and tyrosol) (Soler-Rivas *et al.*, 2000) and belongs to a very specific group of coumarin like compounds, called secoiridoids, which are abundant in *Oleaceas* family. Secoiridoids, iridoids and demethyloleuropein are compounds bound to glycoside (Bianco *et al.*, 1993; Damtoft *et al.*, 1995). Oleuropein glycoside, aglycon and elenolic acid derivtized from the hydrolysis of oleuropein, as well as aglycons of ligstroside and of oleuropein (Montedoro *et al.*, 1992; Angerosa *et al.*, 1996). Phenolic glucosides include verbascoside which is a heterosidic ester of caffeic acid and hydroxytyrosol (Romani *et al.*, 1999; Owen *et al.*, 2000). Hydroxytyrosol and tyrosol are the main phenolic compounds in extra-virgin olive oil (Chimi *et al.*, 1991; Servili *et al.*, 1999). Olive oil hydrophilic extracts contain many phenolic compounds and phenyl-alcohols; secoiridoid compounds identified in olive oil include 3,4-dihydroxyphenylethanol, 4-hydroxyphenylethanol, 4-hydroxyphenylacetic acid, 3,5-dimethoxy-4-hydroxybenzoic acid, 4-hydroxybenzoic acid, 4-hydroxy-3-methoxybenzoic acid, 2-hydroxycinnamic acid, 4-hydroxycinnamic acid, 3,4-

dihydroxyphenylacetic acid and isomers of oleuropein (Amiot *et al.*, 1986; Amiot *et al.*, 1989; Perrin, 1992; Brenes-Balbuena *et al.*, 1992; Bianco *et al.*, 1993). Oleuropein, hydroxytyrosol and their derivatives are found in large quantities in olive leaves and olive fruits (Tsimidou *et al.*, 1992; Servili *et al.*, 1999). Figures 2.2A and 2.2B show structures of some phenolic compounds in olive meal. Bianco *et al.* (2001) reported the presence of hydroxyisochromans and their derivatives in olives.



Phenolic Compounds	X	Y
Oleuropein	OH	CH_3
Demethyloleuropein	OH	H
Ligstroside	H	CH_3

Figure 2.2A: Chemical structures of some phenolic compounds in olive meal (Shahidi and Naczk, 2004).

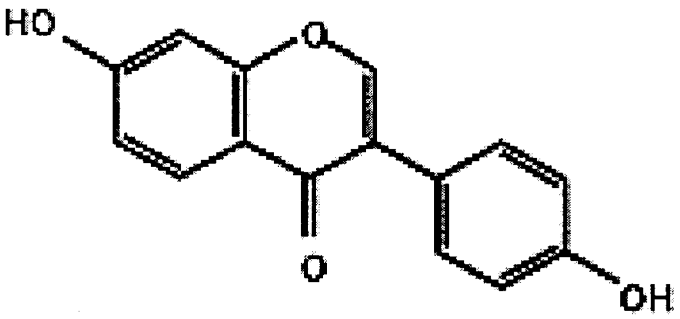


Phenolic Compounds	X	Y
Tyrosol	H	OH
Hydroxytyrosol	OH	OH
Verbascoside	OH	Rhamnose

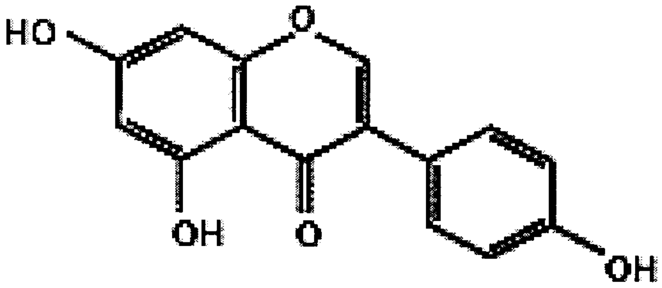
Figure 2.2B: Chemical structures of some phenolic compounds in oil-bearing plants (Shahidi and Naczk, 2004).

2.1.2 Polyphenol Compounds

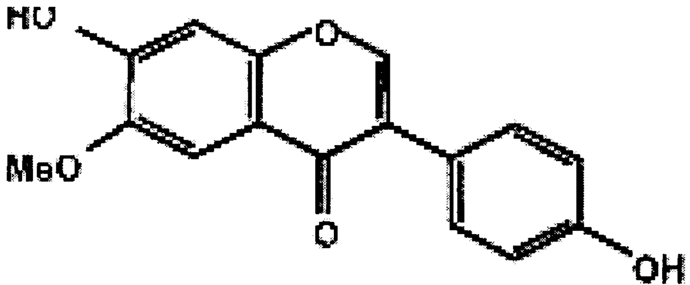
Condensation of more than one unit of phenylpropanoides (C6-C3) leads to formation of flavonoids that has the basic skeleton of diphenylpropanes (C6-C3-C6), which include flavones, isoflavones and anthocynidins (Shahidi and Naczk, 2004). The total content of flavonoids in flaxseed is 35-71 mg/100g (Oomah *et al.*, 1996); the major flavonoids in flaxseed are C-glycosides and O-glycosides (Ibraham and Shaw, 1970). Huang *et al.* (1979) reported that the isoflavone group consists of daidzein (7, 4'-dihydroxyisoflavone), glycitein (7,4'- dihydroxy-6-methylisoflavone) and genistein (6,7,4' trihydroxyisoflavone) (Figure 2.3). These daidzein, glycitein and genistein isoflavones are found in soybean as glucosides, malonylglucosides (malonyldaidzein, malonylglycitein and malonylgenistein) and acetylglucosides, (acetyldaidzein, acetylglycitein and acetylgenistein), isoformononetin, trihydroxyisoflavone and dihydrodaidzin and dihydrogenistein (Gyorgy *et al.*, 1964; Ingham *et al.*, 1981; Kudou *et al.*, 1991; Barnes *et al.*, 1994; Hoeck *et al.*, 2000; Hosny and Rosazza, 2002). The total content of soybean isoflavones is 47.2-420 mg/100 g (Wang and Murphy, 1994; Wang and Murphy, 1996; Simonne *et al.*, 2000). A major anthocyanin known as cyanidin-3-glucoside was identified by Kuroda and Wade (1993) in soybean.



Daidzein



Genistein

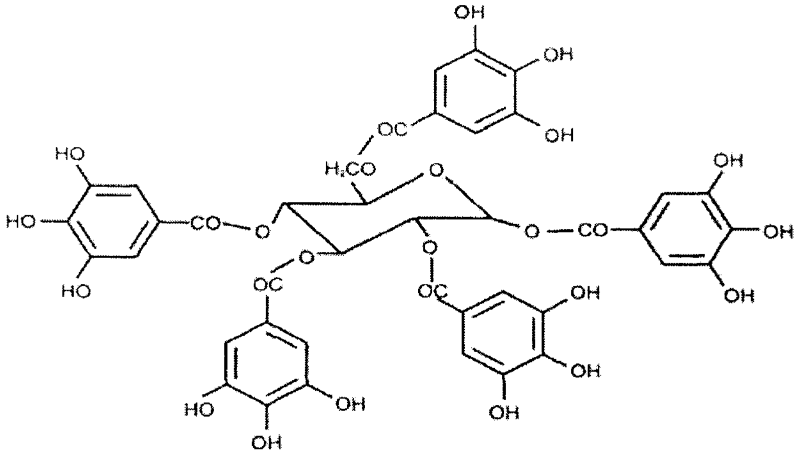


Glycitein

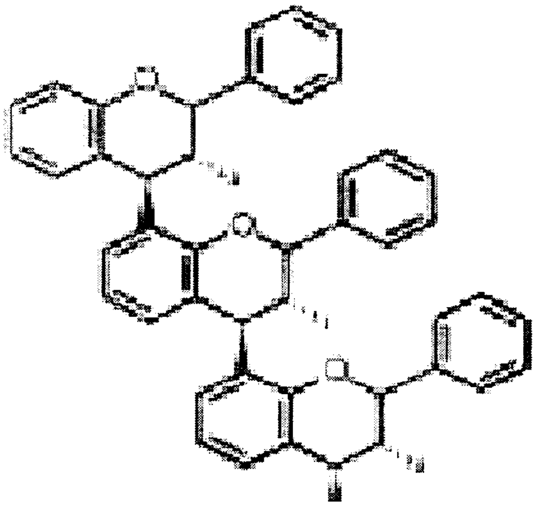
Figure 2.3: Structures of soybean isoflavones (daidzein, glycitein and genistein) (Shahidi and Naczk, 2004).

Isoflavonoid phytoalexins such as coumestrol have been identified in soybean; the coumestrol content of soybean is 0.12-7.11 mg/100 g (Knuckles *et al.*, 1976). The content of polyphenol in olive fruits were 80 mg/100 g (Visioli *et al.*, 2000) depending on cultivar, season, location and degree of ripening (Amiot *et al.*, 1986; Caponio *et al.*, 2001). Flavonoids identified in olive and olive oil include rutin, quercetin, luteolin-7-glucosides and apigenin glucosides (Amiot *et al.*, 1986; Rovellini *et al.*, 1997; Vlahov, 1992; Brenes-Balbuena *et al.*, 1999).

Tannins have been classified according to their chemical structure into hydrozable tannins (tannic acid) and condensed tannin (proanthocyanidins). Hydrozable tannins consist of gallic acid and its dimeric condensation product, hexahydroxydiphenolic acid esterified to a polyol, which is mainly glucose (Figure 2.4); it is a gallotannin consisting of a pentagalloyl glucose molecule that can further be esterified to form five gallic acid units (Porter, 1989). Condensed tannins have been identified in rapeseed hulls (Bate-Smith and Ribereau-Gayon, 1959). The content of tannins in rapeseed meal is 3% (Clandinin and Heard, 1968). Blair and Reichert (1984) reported that the content of tannins is 0.09%-0.39% in defatted rapeseed catyledon and 0.23%-0.54% in defatted canola catyledon. Canola meal contains 0.68%-0.77% condensed tannins (Shahidi and Naczk, 1988; 1989) while rapeseed hull contains 0.02%-0.22% condensed tannins (Leung *et al.,* 1979). The total tannins content in rapeseed/canola hull is 0.14-23 g/kg; the crude tannin extract from canola hull contains 20% proanthocyanidins of the total tannins content (Naczk *et al.*, 1994).



Tannic Acid

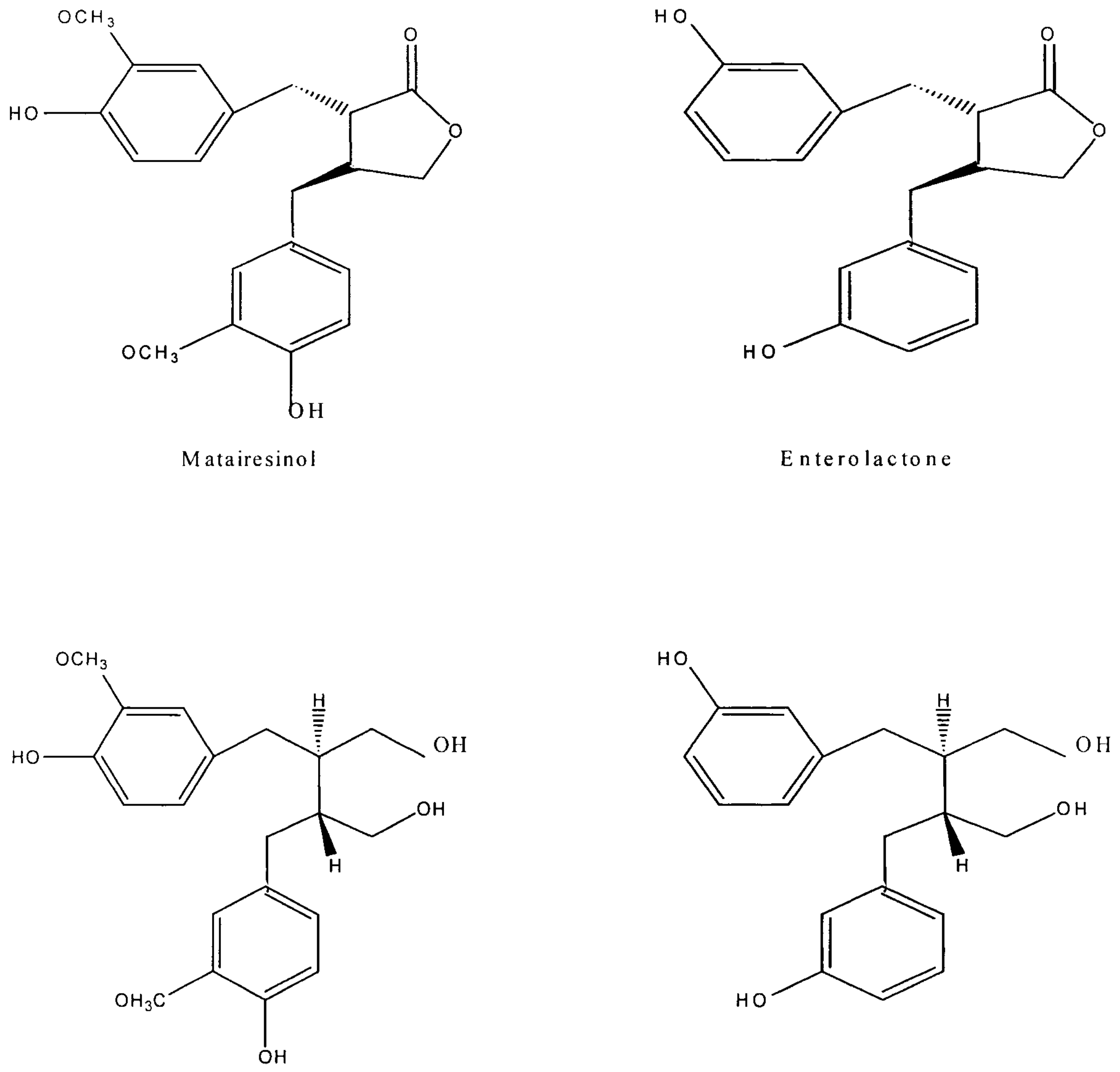


Condensed Tannin

Figure 2.4: Structures of tannic acid and condensed tannin found in oil-bearing plants (Bravo, 1998).

2.1.3 Lignans

Lignans are phytochemicals that are formed from combination of two units of phenylpropanoids (C6-C3) linked by a central carbon of their side chain in plant cell wall; they are been classified into four major groups; lignans, lignolides, monoepoxylignans and biepoxylignans (Shahidi and Naczk, 2004). Lignan has multi-functional properties with important roles in plant defense against bacteria, viruses, fungi and toxic microorganisms (Davin and Lewis, 1992; Lewis and Yamamoto, 1990; Douglas, 1996). Flaxseed is considered as a good source of lignan with more than 800 times than in any other plant (Pszczola, 2002) with 0.68 mg/g in defatted flaxseed meals (Thompson, 1994). Secoisolariciresinol and other phenolic compounds in flaxseed are found in bound forms with both glucosidic and ester (Bambagiotti-Alberti *et al.*, 1994; Westcott and Muir, 1996; Chimichi *et al.*, 1999). Plant lignans such as matairesinol and secoisolariciresinol diglucoside (SDG) have been shown to be the precursor for mammalian lignans which are generated in the colon by the action of bacterial flora (Thompson *et al.*, 1991). SDG is a major lignan components; defatted flaxseed contains 1%-4% lignans (Bakke and Klosterman, 1956; Johnsson *et al.*, 2000). The most important mammalian lignans enterolactone and enterodiol which are generated by the removal of two methyl groups and two hydroxyl groups from matairesinol and SDG, respectively (Borriello *et al.*, 1985; Qiu *et al.*, 1999). Matairesinol is present in flaxseed in relatively low content compared to SDG (Mazur and Adlercreutz, 1998). Other lignans found in flaxseed include SDG isomer, isolariciresinol and pinoresinol diglucoside (Bambagiotti-Alberti *et al.*, 1994; Meagher *et al.*, 1999; Qiu *et al.*, 1999). Lignans reported in olive oil such as pinoresinol, 1-acetoxypinoresinol and pinoresinol diglucoside.



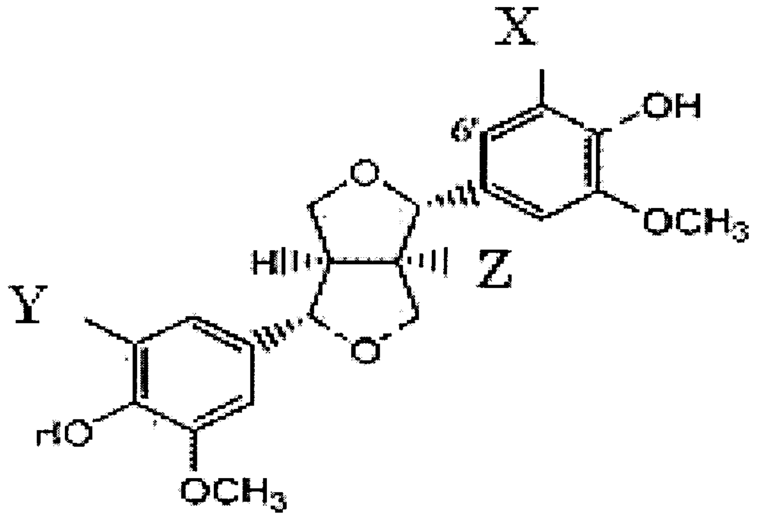
Matairesinol

Enterolactone

Secoisolariciresinol

Enterodiol

Figure 2.5A: Structures of lignans (secoisolariciresinol, matairesinol, enterolactone and enterodiol) in flaxseed (Meagher *et al.*, 1999).



Compounds	X	Y	Z
Pinoresinol	H	H	H
Acetoxypinoresinol	H	H	$COOCH_3$

Figure 2.5B: Structures of lignans (pinoresinol and acetoxypinoresinol) in olive oil (Owen *et al.*, 2000).

The content of pinoresinol and 1-acetoxypinoresinol are 5-67 mg/kg in olive oil (Qiu *et al.*, 1999; Brenes- Balbuena *et al.*, 1999). Figure 2.5A and 2.5B show the structures of lignans in oil-bearing plants.

2.2 Phenolic Compounds Interactions with Food Components

2.2.1 Protein-Phenolic Interactions

Procyanidin compounds are a class with great structural diversity that has demonstrated a specific affinity for proteins (Okuda *et al.*, 1985; Ricardo da Silva *et al.*, 1991). The interactions between proteins and procyanidins are affected by the solvent composition, ionic strength, pH and temperature (Goldstein and Swain, 1965; Asano *et al.*, 1982; Asquith and Butler, 1986). Galloyl-D-glucose has shown ability to associate with bovine serum albumin (BSA); this ability is enhanced in the presence of galloyl ester group (Beart *et al.*, 1985). Dietary tannin has ability to precipitate proteins from aqueous solution (Mehansho *et al.*, 1983; Baxter *et al.*, 1997). Soybean proteins have been shown to interact with chlorogenic, caffeic acid, cinnamic and gallic acids and the flavonoids, flavone, epigenin, kaempferol, quercetin and myricetin; these interactions lead to reduced nutritional value of lysine, cystein and tryptophan residues in the soybean proteins (Wang and Murphy, 1994).

The formation of tannin-protein complexes depends on the size, conformation and charge of the protein molecules, and size, length, and flexibility of tannins (Shahidi and Naczk, 1995). Globular structures of proteins such as ribonuclease, lysozyme and cytochrome C have low affinity to bind with tannins, while open structures of proteins (gelatin) have high affinity to bind with tannins (Hagerman and Butler, 1981). Artz *et al.*

(1987) demonstrated that tannins need at least three flavanol subunits to be effective in precipitating proteins. Tannins are less effective precipitating agents while simple flavanols do not precipitate protein (Artz *et al.*, 1987). Tannins or their oxidized forms interact with essential amino acids and other nutrients (Ohlson and Anjou, 1979). The precipitation of tannin-protein complex was pH sensitive (Hagerman and Butler, 1978). Naczk *et al.* (1996) reported that the solubility of tannin-protein complex was the lowest at pH 0.3-3.1, while bovine serum albumin (BSA), fetuin, collagen and pepsin were precipitated at pH 3.0-5.0.

The interactions of simple phenolic compounds with proteins in model systems have been investigated, including interaction between sinapine and bovine serum albumin (BSA); phenolic-protein complexes formation was favored in neutral and basic pH conditions (Smyk and Drabent, 1989). Zadernowski (1987) reported that BSA binds with the ester bond of sinapine. Condensed tannins extracted from canola hull precipitated 3.0-59.0 mg BSA/g hulls (Leung *et al.*, 1979). In mammals, interaction of proline rich salivary proteins with dietary tannins results in the astringent taste (Mehansho *et al.,* 1987; 1994; Baxter *et al.*, 1997).

Tannins interact with proteins due to the chemical and structural properties of the proteins and tannins; the hydrophobic interactions (between the aromatic ring of tannin and the hydrophobic region of proteins) and hydrogen bonds (Oh *et al.*, 1980; Hagerman and Butler, 1980; McManus *et al.*, 1985; Hagerman, 1989). Many vegetable protein products can bind to phenolic compounds through different mechanisms such as hydrogen bonding, covalent bonding, ionic bonding, hydrophobic interactions and electrostatic interaction (Mason, 1955; Loomis and Battaile, 1966; Loomis, 1974; Hagerman and Butler, 1978;

Rubino *et al.*, 1996). Rubino *et al.* (1996) suggested that thomasidioic acid bind with canola proteins at pH 7.0 and 8.5 with hydrophobic interactions.

Proteins interact also with phenolic compounds by hydrogen bonding via hydroxyl groups in phenolic compounds and carbonyl groups of the peptide bonds, or due to the oxidation to quinines that bind with reactive groups of proteins (Loomis and Battaile, 1966). Shahidi and Naczk (1995) reported that the phenolic-protein complex form at both low and high concentrations of proteins due to the formation of hydrophobic interactions. Butler *et al.* (1984) suggested that the phenolic compounds and proteins do not interact through ionic binding at physiological pH.

The interaction between polyphenol with polypeptides responsible for haze or chill phenomena occur via haze active (HA) sites; the haze phenomena have been found in beer, wine and juice such as grapes and apples and it is desirable to remove haze-active site material (Goertges, 1982; Heatherbell, 1976; Hough *et al.*, 1982; Siebert *et al.*, 1996). The main component of chill and permenant hazes is polypeptides, polyphenols and minor amount of metals (Gramshaw *et al.*, 1970; Djurtoft, 1965). The major phenolic compounds identified in haze is anthocyandins such as procyanidin, prodelphinidin, and propelargonidin (Bengough and Harris, 1955; McFarlane *et al.*, 1955), lignin and phenolic acids include ferulic, sinapic, vanillic, syringic, gallic, protocatechuic, and caffeic acids (Harris, 1965; Harris and Ricketts, 1959). The major components of chill haze are polyphenol compounds, while the permanent haze is condensed tannins.

Ali (2002) reported that the thermal stability of BSA proteins was affected by the presence of gallic acid at pH 7, and the thermal stability of proteins increased as a results of interaction with phenolic compounds; the interactions were affected by temperature and pH (Ali, 2002).

2.2.2 Carbohydrate-Phenolic Interactions

Phenolic-carbohydrate complexes are common in most plant foods such as fruits, legumes, cereals, oil-bearing plants and beverages. The chemical nature of phenolic compounds allows them to conjugate covalently with monosaccharide, disaccharides and oligosaccharides (Bravo, 1998). Glucose is the most common sugar associated with phenolic compounds. Galactose, rhamnose, xylose, arabinose, glucuronic acid and galacturonic acid are also common phenolic compounds (Bravo, 1998). Generally, phenylpropanoids and simple phenolic compounds such as hydroxybenzoic acid and benzenaldehyde derivatives are covalently linked with polysaccharides in cell walls (Wallace *et al.*, 1991). Naringenin and hesperidin can occur either as O-glycosides or C-glycosides and are common found in citrus foods and prunes (Herrmann, 1988).

Anthocyanin includes the glycosides group such as pelargonidin, malvidin and cyaniding; in addition to glycosylation, common linkages occur with aromatic and aliphatic acids as well as methyl ester derivatives (Mazza, 1995). Simple phenolic compounds and flavonoids represent the majority of plant phenolic compounds. Some of these phenolic compounds can be linked to cell wall components with an ester linkage such as polysaccharides, due to the chemical nature of these compounds which can be extracted from fiber matrix with alkaline hydrolysis (Bravo, 1998). Non-extractable polyphenolic compounds that have high molecular weight and some simple phenolic compounds bound with dietary fiber remain insoluble in organic solvents (Bravo *et al.,* 1994; Saura-Calixto and Bravo, 1995).

2.2.3 Lipid-Phenolic Interactions

Suberins and cutins are considered as class of polyphenolic compounds composed of polymerization more than unit of phenylpropanoids with long chain fatty acids or fatty alcohols (18-30 carbon atoms) or hydroxyl fatty acid and dicarboxylic acids with 14 to 30 carbon atoms which is the main constituents of cell wall (Davin and Lewis, 1992). Balde *et al.* (1991) has been reported that the phenolic acids and their esters with long chain fatty ester are found in the bark of stem. Propolis is composed of esters of phenolic acids and their derivatives with fatty alcohols (Banskota *et al.*, 2001; Castaldo and Capasso, 2002). Chemical synthesis of benzoic and phenolic acids to produce phenolic-lipid esters have been investigated using acidic catalysts, basic catalysts and lipase catalysts (Gutman *et al.*, 1992; Humeau *et al.*, 1995). The esterification of cinnamic and *p*-hydroxybenzoic acids with short or medium chain fatty acid using lipase have been reported by Stamatis *et al.* (1999). Several authors have reported on the esterification of phenolic acids using long chain fatty alcohols to produce phenolic-lipid compounds (Lue *et al.*, 2005). The synthesis of a scorbyl palmitate using novozym 435 produce from using ascorbic acid with palmitic acid methyl ester or palmitic acid has been reported (Humeau *et al.*, 1995). Transesterification of ethylferulate with triolein in toluene using novozym 435 produce feruyl monoolein and feruyl diolein has been reported by Compton *et al.* (2000). Karboune *et al.* (2005) reported that transesterification of cinnamic acid with triolein in organic solvent media produce a combined of monoley-1(3)-cinnamate and dioleyl-2-cinnamate.

The esterification of linoleyl alcohol with dihydrocaffeic acid in hexane medium was investigated by Sabally (2006).

2.2.4 Mineral-Phenolic Interactions

Formation of tannins–metal complexes has an important role against microorganisms (Scalbert, 1991; Mila and Scalbert, 1994). Many authors suggested the use tannins as modifier for rheological properties of minerals and clays (chelators of minerals from waste water and components of anticorrosive primers and inks (Seavell, 1978; Grimshaw, 1976; Randall *et al.*, 1974). Simple phenolic compounds were used to precipitate copper and zinc in model system; it is reported that phenolic-metal complexes is affected by concentration of metal and phenolic compounds (McDonald *et al.*, 1996).

2.3 Properties of Food Phenolic Complexes

2.3.1 Health Benefits

Consumption of oilseeds has been reported to have positive effect on breast, prostate and colon cancers (Martein Moreno *et al.*, 1994; Keli *et al.*, 1996; Lipworth *et al.*, 1997). Many studies have illustrated that oleuropein, verbascoside and tyrosol have anti-tumoral properties and positive effects on myocardial vascular disease (Petroni *et al.*, 1995; Pennacchio *et al.*, 1996; Maimeskulova and Maslov, 1998; Lee *et al.*, 1993; Saracoglu *et al.*, 1995; Saenz *et al.*, 1998). Oil-seeds containing polyphenolic compounds can reduce oxidation of low density lipoprotein (LDL) (Wiseman, 1996). Polyphenolic compounds showed hypoglycemic effect (Trovato *et al.*, 1993). In addition to their oxidative stability (Tsimidou *et al.*, 1992), several studies have demonstrated the capacity of phenolic compounds to scavenge peroxy radicals, hydroxy radicals, and superoxide anion and the chelation of transition metal ions (Chimi *et al.*, 1991; Visioli and Galli, 1994; Chimi *et al.*, 1995; Visioli and Galli, 1995; Assmann *et al.*, 1997).

Flaxseed is one of many plant species that is widely studied for potential human health and nutraceutical materials (Haumann, 1993), it is a natural source of phytochemicals such as flavonoids, coumarins, lignans and phenolic acids (Caragay, 1992). Lignans, SDG and other phenolic compounds in flaxseed are implicated in potential human health such as phytoestrogenic, antioxidant, anti-carcinogenic (breast and prostate) and cardio protective effects (Cunnane and Thompson, 1995; Oomah and Mazza, 1998; Westcott and Muir, 2000; Clifford, 2000; Anonymous, 2001). Lignans have also been shown to stimulate synthesis of sex hormone binding globulin, that improves the clearance of circulating estrogen (Pszczola, 2001). Enterolactone and enterodiol were found in the urine during reproductive cycle of pregnancy and it has negative relationship with oestrogen (Setchell *et al.*, 1983). Many authors suggested that the lignans play an important role in hormone metabolism (Setchell *et al* 1983; Tham *et al.*, 1994). Lignin has been suggested to play an important role in reducing breast cancer (Dai *et al.*, 2002; Ingram *et al.*, 1997; den Tonkelaar *et al.*, 2001). Dietary lignans have an important role in preventing prostate and colon cancer (Lin *et al.*, 2001; Jenab and Thompson, 1996).

Simple and complex phenolic compounds in soybean have been studied extensively in relation to their estrogenic, anti-cancer, anti-mutagenic and anti-oxidants roles (Makela *et al.*, 1995; Wiseman; 1996; Kuntz *et al.*, 1999; Miyazawa *et al.*, 2001; Wei *et al.*, 1995; Arora *et al.*, 1998); soybean isoflavones and other phenolic compounds have been reported to be anticancerogenic for different kind of cancers (Messina *et al.*, 1994; Akiyama and

Ogawara, 1991; Kiguchi *et al.*, 1990; Okura *et al.*, 1988; Schweïgerer *et al.*, 1992; Watanabe *et al.*, 1991) including breast cancer (Folman and Pope, 1966; Makela *et al.*, 1995) and prostate cancer (Severson *et al.*, 1989). Some studies have reported that consumption of soybean products has beneficial effects on human health against myocardial vascular disease via the phenolic compounds induced reduction of low density lipoproteins (LDL) and increase of high density lipoproteins (HDL) (Hodges *et al.*, 1967; Nilausen and Meinertz, 1998; Potter *et al.*, 1996).

2.3.2 Antimicrobial Effects

Many authors have reported that polyphenol compounds have antimicrobial effects against *Staphylococcus aureus*, *Salmonella enteritidis* and *Bacillus cereus T* spores (Walter *et al.*, 1973; Tranter *et al.*, 1993; Tassou and Nychas, 1994; Tassou and Nychas, 1995). The phenolic compounds inhibit growth and delay formation of sporulation produced by *Aspergillus parasiticus* (Gourama and Bullerman, 1987). It has been reported that different classes of phenolic compounds inhibit growth of wide range groups of gram-positive and gram-negative bacteria (Fleming *et al.*, 1973; Beuchat and Golden, 1989). Several studies has reported that phenolic compounds such as *p*-hydroxybenzoic acid, vanillic acid, and *p*–coumaric acid inhibit the growth of *Escherichia coli*, *Klebsiella peneumoniae* and *B cereus* (Calis *et al.*, 1988; Pardo *et al.*, 1993; Aziz *et al.*, 1998).

Phenolic compounds have been studied as antiviral activity against human respiratory Syncytial virus (Chen *et al.*, 1998; Kernan *et al.*, 1998). Capasso *et al.* (1995) reported that the presence of polyphenolic compounds and phenolic acid inhibit growth both of phytopathogenic *Pseudomonas syringae*, *Pv savastanoi* and *Corynebacterium michiganense* (Kernan *et al.*, 1998).

2.3.3 Nutritional and Physiological Properties

Phenolic compounds can reduce digestibility and absorption of proteins, lipids and carbohydrates (Bressani *et al.*, 1988; Butler, 1989; Longstaff and McNab, 1991; Alzueta *et al.*, 1992; Longstaff *et al.*, 1993; Tanner *et al.*, 1994; Quesada *et al.*, 1996; Bravo and Saura-Calixto, 1998). Many studies have reported the inhibition of amylolytic enzymes and reduction in digestibility of dietary carbohydrates and decrease in glycemic response (Thompson *et al.*, 1984). Polyphenolic compounds and condensed tannins increased fecal fat excretion (Martin-Carron *et al.*, 1997).

Some phenolic compounds with galloyl and catechol groups have been shown to have a negative effect on cation absorption (Brune *et al.*, 1989). Flavonoids such as catechins, phenolic acids such as chlorogenic acid and polymerized products lead to reduce iron bioavailability (Brune *et al.*, 1989; Cook *et al.*, 1995; South *et al.*, 1997; Hurrell *et al.*, 1998). Garcia-Lopez *et al.* (1990) reported that there is no effect of tannins from soybean, chickpeas and red kidney beans on iron absorption. Kies and Umoren (1989) reported reduction in copper absorption after consumption of tea, while Vaquero *et al.* (1994) reported an increase in absorption of copper after consumption of tea.

The negative effects of polyphenolic compounds on bioavailability of metals such as copper and iron are considered to be related to hydroxyl radical production (Thompson *et al.*, 1976); chelating of these metals is one way by which polyphenolic compounds exert their antioxidant activity. Coudray *et al.* (1999) reported that the presence of chlorogenic and caffeic acids reduce zinc absorption in rates. Often reports suggest negative effects of polyphenolic compounds on bioavailability of sodium (Freeland *et al.*, 1985) and aluminum (Fairweather-Tait *et al.*, 1991), but no effect on manganese, calcium, and magnesium (Fraile and Flynn, 1992; Jansman *et al.*, 1993).

2.3.4 Antioxidant Properties

The roles of phenolic compounds as antioxidants to maintain the quality of food and to prevent or delay deterioration and maintain the nutritional value of food and food products, and to protect body tissues from oxidative damage have been described (Halliwell, 1999; Shahidi, 2000). Phenolic compounds have different mechanisms for their action as antioxidants; these include reduction of oxygen concentration, terminators of free radicals, decompose primary products of oxidation to non-radical species, and prevent continued hydrogen abstraction from substrate and chelators of metal ions that are lead to form peroxidation compounds (Shahidi and Naczk, 2004). The chemical nature of phenolic compounds as antioxidants in preventing oxidation by donation of a hydrogen atom to radicals has also been described (Shahidi, 2000).

The chemical structure of polyphenol compounds with higher molecular weights allow them to have higher antioxidants capacity as compared to simple phenolic compounds due to the presence of hydrogen atoms with ethyl or *n*-butyl substitute groups (Shahidi *et al.*, 1992). Ratty and Das (1988) reported high antioxidant capacity of foods that contain flavonoids due to increase in the degree of hydroxylation. Polyphenol compounds inhibit reactions of active oxygen radicals that cause oxidation (Fuhrman *et al.*, 1995). Afanas'ev *et al.* (1989) reported that polyphenol compounds prevent formation of first chain reaction as terminator for initial radicals such as hydroxyl and metal ions that form complex with metal ions. SDG and secoisolariciresinol (SECO) have been identified to have antioxidant properties (Kitts *et al.*, 1999; Prasad, 2000; Niemeyer and Metzler, 2002). Table 2.4 shows the antioxidant compounds present in oil-seeds.

Murakami *et al.* (1984) reported that daidizein, genistein and glycitien have antioxidant activities. Aglycons have superior antioxidant activity compared to the parent isoflavone glycosides (Hayes *et al.*, 1977). Syringic, vanillic, caffeic, ferulic, *p*-coumaric and *p*-hydroxybenzoic acids in soybean have shown antioxidant activity (Arai *et al.*, 1966; Hammerschmidt and Pratt, 1978; Pratt and Birac, 1979). The order of antioxidant activity of soy isoflavone is glycetin>diadzein>genistein>quercetin> 6,7,4'-trihydroxyisoflavones, while for phenolic acid the order is *p*-coumaric acid>ferulic acid> chlorogenic acid> caffeic acid (Arai *et al.*, 1966; Hammerschmidt and Pratt, 1978; Pratt and Birac, 1979).

Oleuropein a hydroxytyrosol derivative which occurs in large amounts in olive leaves and olive fruits, has weak antioxidant activity (Tsimidou *et al.*, 1992). It has been reported that virgin olive oils with high polyphenol content are more resistant to auto-oxidation; these compounds have high activity on the auto-oxidative stability of olive oil (Montedoro *et al.*, 1992). Hydroxytyrosol and their derivatives have also shown antioxidant activity (Chimi *et al.*, 1991; Montedoro *et al.*, 1993).

Table 2.4: Antioxidant activity of phenolic compounds identified in oil-seeds.

Oil-seed	Phenolic Compounds with Antioxidant activity
Rapeseed, canola[1]	sinapine, benzoic and cinnamic acid derivatives, phenolic acid esters and glycosides.
Mustarseed[2]	sinapine, ester of phenolic acids.
Soybean[3]	syringic, ferulic, vanillic, salicylic, *p*-coumaric acids and esters, chlorogenic, caffeic, sinapic acids, isoflavones and their glucosides.
Peanut[4]	phenolic acids and esters; *p*-hydroxybenzoic, *p*-coumaric, syringic, ferulic, caffeic acids.
Sunflower seed[5]	chlorogenic, caffeic, *p*-hydroxybenzoic, *p*-coumaric, cinnamic, *m*-hydroxybenzoic, vanillic, gallic, syringic acids, catechin, epicatechin.
Flaxseed[6]	*p*-hydroxybenzoic, coumaric, ferulic, sinapic acids, lignans, and their glucosides.
Cottonseed[7]	*p*-hydroxybenzoic, ferulic, sinapic acids, quercetin , rutin.
Sesame seed[8]	vanillic, coumaric, ferulic, sinapic acids, lignans.
Olive fruits[9]	tyrosol, hydroxytyrosol, secoiridoids, flavonoids, lignans.

Sources: (1) Dabrowski and Sosulski (1984), Kozlowska *et al.* (1984), Krygier *et al.* (1982), Amarowicz *et al.* (2000); (2) Dabrowski and Sosulski (1984), Amarowicz *et al.* (1996), Henning (1982), Krygier *et al.* (1982); (3) Walter (1941), Amarowicz *et al.* (1996), Arai *et al.* (1966), Hoppe *et al.* (1997), Murakami *et al.* (1984); (4) Henning (1982); (5) Leung *et al.* (1981), Sabir *et al.* (1974); (6) Amarowicz *et al.* (1996), Henning (1982), Amarowicz and Shahidi (1994), Amarowicz *et al.* (1994), Johnsson *et al.* (2000); (7) Hron *et al.* (1999), Henning (1982), Amarowicz *et al.* (1996); (8) Henning (1982), Amarowicz *et al.* (1996), Fukuda *et al.* (1986); (9) Gutfinger (1981), Gennaro *et al.* (1998).

CHAPTER 3

PHENOLIC COMPOUNDS IN FLAXSEED, SOYBEAN AND OLIVE

3.1 Justification

Phenolic compounds are now recognized as important minor component of many edible plants, including many oil-bearing plants such as soybean, canola, flaxseed and olive that are used as food or sources of food ingredients. The types, amount and properties of phenolic compounds in these foods vary tremendously. This chapter addresses the first objective, which is to investigate the distribution and antioxidant activity of free and bound phenolic compounds in flaxseed, soybean and olive. The chemical structure of phenolic compounds allow them to be found either free or bound with other components such as proteins, carbohydrates and lipids. Free phenolic compounds were fractionated on the basis of their solubility characteristics in water and methanol. Dilute base and dilute acid were used to liberate the phenolic compounds bound to other components followed by methanol extraction. Reversed phase high pressure liquid chromatography (RP-HPLC) and mass spectrometry (MS) were used for identification of individual components of phenolic compounds. Antioxidant Activity (AA%) of free and bound phenolic compounds was measured using linoleic acid/β-carotene assay.

3.2 Materials and Methods

3.2.1 Materials

Soybean (Cultivar SB, 210) was obtained by Great Lakes Organic Inc. (Ontario, Canada). Flaxseed was obtained from La Meunerie Milanaise Inc. (Milan, PQ, Canada). Olive samples were obtained from Jordan (Irbid, Rahoub Valley); the samples were collected during 2003 at black maturation, fruits were immediately frozen then the fleshy part was separated from the olive stone. Samples were defatted using petroleum ether using a Soxhlet apparatus for 10 h (AOAC, 1990).

3.2.2 Extraction of Phenolic Compounds

3.2.2.1 Methanol and Water Extractions

A sample (1 g) was extracted using 25 ml 100% methanol for 1 h at 24°C in a water bath then centrifuged (10,000 X g, 10 min). The methanol supernatant was separated and the residue was extracted with 25 ml water (24°C/4 h) followed by centrifugation (10,000 X g, 10 min). Total phenolic content was determined for each extract. The phenolic compounds obtained by this technique were designated as methanol extraction and water after methanol extraction.

3.2.2.2 Water and Methanol Extractions

A sample (1 g) was extracted using 25 ml distilled water for 1 h at 24°C in a water bath then centrifuged (10,000 X g, 10 min). The water supernatant was separated and the residue was extracted with 25 ml 100% methanol (24°C/4 h) followed by centrifugation (10,000 X g, 10 min). Total phenolic content was determined for each extract. The phenolic compounds obtained by this technique were designated as water extraction and methanol after water extraction.

3.2.2.3 Methanol Extraction

A sample (1 g) was extracted with 25 ml 100% methanol (24°C/1 h) then centrifuged (10,000 X g, 10 min). Using methanol extraction for phenolic compounds has been reported by Van Ruth *et al.* (2001). The supernatant was filtered using cheese cloth then flushed under a stream of nitrogen and stored at –18°C for further analysis.

The residue remaining from methanol extraction described above was extracted with 25 ml 100% methanol (60°C/1 h) and centrifuged (10,000 X g, 10 min). Use of heat for phenolic compounds extraction has been reported by Johnsson *et al.* (2000). The supernatant was filtered using cheese cloth then flushed under a stream of nitrogen, and stored at –18°C for further analysis. The phenolic compounds obtained by this technique were designated as free phenolic compounds. Total phenolic content was determined in the extracts.

3.2.2.4 Alkaline/Acid Hydrolysis

Base hydrolysis to extract bound phenolic compounds has been reported by Krygier *et al.* (1982) and Eliasson *et al.* (2003). The residue remaining from methanol/heat extraction described in Section 3.2.2.3 was hydrolyzed with dilute alkaline solution (25 ml, pH 12.0, 0.1 N NaOH) for 12 h at 24°C then centrifuged (10,000 X g, 10 min). The supernatant was filtered with cheese cloth. The bound phenolic compound of lyophilized supernatant extracted with 25 ml 100% methanol (23°C/1 h) and centrifuged (10,000 X g, 10 min). The supernatant was filtered using cheese cloth then flushed under a stream of nitrogen and stored at –18°C for further analysis.

The residue remaining from alkaline extraction described above was hydrolyzed with dilute acid solution (25 ml, pH 2.0, 0.1 N HCl) for 12 h at 24°C. Several authors used dilute acid to extract bound phenolic compounds (Fernandez-Bolanos *et al.*, 2001; Eliasson *et al.*, 2003). The supernatant was filtered with cheese cloth. The bound phenolic compound of lyophilized supernatant was extracted with 25 ml 100% methanol (23°C/1 h) and centrifuged (10,000 X g, 10 min). The phenolic compounds obtained by this technique were designated as bound phenolic compounds. Total phenolic content was determined in the extracts.

3.2.3 Determination of Total Phenolic Content

The total phenolic content of each extract obtained in the Section 3.2.2 was determined by the Folin-Ciocalteu spectrophotometric method (Hoff and Singleton, 1977) with modifications; a standard curve was prepared using a stock solution of gallic acid (50 mg/50 ml). 10 ml of the extract containing phenolic compounds was first diluted to 50 ml with distilled water; 1 ml was taken for analysis, 7.5 ml of distilled water was added, followed by 0.5 ml Folin-Ciocalteu reagent. After 4 min, 1 ml of 5% sodium carbonate (Na_2CO_3) was added. The contents were mixed, and after 1 h the absorbance of the green color was measured at 725 nm.

3.2.4 HPLC Analysis of Phenolic Compounds

The phenolic extracts obtained in the Section 3.2.2 were evaporated under a stream of nitrogen then dissolved in 1 ml methanol. The sample was mixed, centrifuged (5,000 X g, 10 min), flushed under a stream of nitrogen and stored at –18°C for HPLC analysis. Standards of phenolic compounds (5 μg) were dissolved in methanol (1 ml) followed by flushing under a stream of nitrogen and stored at –18°C. RP-HPLC analysis was carried out according to the method described by Deslauriers (2000) using a Beckman (CA, USA) liquid chromatography equipped with a Programmable Solvent Module (model 126) solvent delivery system, a Beckman Manual injector a (20 μl loop for sample, 100 μl for standard) and a Programmable Detector Module (Model 166). Spectral and chromatographic data were analyzed by the Gold System (version V810), translated into PRN format for Microsoft Excel manipulation and stored on disc. For chromatographic separation, the samples were injected into a Zorbax SB-C_{18} reversed phase column (pore size 5 μm, 250 X 4.6 mm i.d, USA) operated at room temperature. Elution was carried out at a flow rate of 0.75 ml/min with the following two-buffer gradient system: solvent A, 0.2% TFA in water (V/V); solvent B, 100% methanol, with a linear gradient starting at 5% to 80% methanol in 50 min, the initial conditions were then re-established over 10 min. The UV detector was calibrated and the elute was monitored at 280 nm monitoring at 1-second interval.

3.2.5 Mass Spectrometry (MS) Analysis of Phenolic Compounds

Standards of phenolic compounds, and phenolic extracts in the Section 3.2.2 were evaporated under a stream of nitrogen then dissolved in 1 ml of 10% acetic acid and injected into to ESI-MS (Waters Micromass QTOF Ultima Global Micromass; Manchester, UK) hybrid mass spectrometer equipped with a nanoflow electrospray source. The MS was operated in positive ionization mode (+ESI) at 3.80 kV, with source temperature of 80°C and desolvation temperature of 150°C. The TOF was operated at an acceleration voltage of 9.1

kV, a cone voltage 100 V and collision energy of 10 eV (for MS survey). For the MS survey mass range, (m/z) was 400-1990, and scanned continuously over the chromatographic run. The mass spectrometer was tuned and calibrated with (Glu)-Fibrinopeptide B (Sigma Chemical Co.; Louis, MO). Instrument control and data analysis were carried out by MassLynx V4.0 software (Waters Corporation, 2005).

3.2.6 Determination of the Antioxidant Activity

Antioxidant activity (AA%) was determined according to the methods described by Lee *et al.* (1995) and Emmons *et al.* (1999) with modification. 5 mg β-carotene was dissolved in 50 ml chloroform, and emulsion was prepared as follows; 3 ml β-carotene solution, 50 μl linoleic acid, and 400 mg Tween 20 were added to a volumetric flask. Chloroform was removed under a stream of nitrogen, and 100 ml distilled water was added, then the mixture was shaken and saturated with oxygen. 3 ml of the β-carotene/linoleic acid emulsion was mixed with 100 μl sample solution and incubated in a water bath (50°C/60 min). Oxidation of the emulsion was monitored by measuring absorbance at 470 nm over 60 min. Control samples contained 100 μl of solvent instead of sample and water was used as blank. The antioxidant activity is expressed using the following equation:

$$AA\% = 100\,(DR_c - DR_s)/DR_c$$

Where AA% is the antioxidant activity, DR_c is the degradation rate of the control; DR_c= (In (A/B)/60), DRs is the degradation rate in the presence of the sample; DRs= (In (A/B)/60), A is initial absorbance at time 0 min, and B is the absorbance after 60 min.

3.3 Results and Discussion

3.3.1 Phenolic Compounds Distribution

Table 3.1 shows the phenolic compounds content based on sequential extraction with methanol and water. The total phenolic compounds using water extraction followed by methanol extraction were 2.09, 2.32 and 4.48 mg/g for full-fat meals and 2.49, 4.65 and 10.90 mg/g for defatted meals of flaxseed, soybean and olive, respectively. The total phenolic compounds content using methanol extraction followed by water extraction were 3.51, 4.05 and 7.84 mg/g for full-fat meals and 1.87, 2.12 and 7.92 mg/g for defatted meals in flaxseed, soybean and olive, respectively. For flaxseed, 1.17-1.90 mg/g (50%-60% of total phenolic compounds) were extracted by an initial extraction with either water or methanol. With soybean, water extracted more than 1.45-3.05 mg/g (60% of the total phenolic compounds) regardless of whether it was used as extractant before or after methanol; the phenolic compounds of soybean appear to be more water soluble than methanol soluble. For olive meal, water extracted 3.23-9.78 mg/g (80%-95% of total phenolic compounds). Solvents which have been used to solubilize phenolic compounds include water, methanol and acetone; methanol is most frequently used as extracting solvent (Shahidi and Naczk, 2004; Krygier *et al.*, 1982; Naczk *et al.*, 1992). Contents of phenolic compounds in flaxseed, soybean and olive depend on many factors such as ratio of sample to solvent, nature of sample, cultivar, the extraction period, season and type of solvent used for extraction (Antolovich *et al.*, 2000; Shahidi and Naczk, 2004; Obied *et al.*, 2005; Maga and Lorenz, 1974; Dabrowski and Sosulski, 1984; Naczk and Shahidi, 1992; Dabrowski and Sosulski, 1984; Naczk *et al.*, 1992).

Table 3.2 and Figure 3.1 show the distribution of phenolic compounds content for full-fat and defatted flaxseed, soybean and olive meals based on methanol extraction, methanol extraction/heat, alkaline hydrolysis and acid hydrolysis carried out in sequence. For full-fat soybean, flaxseed and olive meals; the combination of methanol and heat extracted approximately 90% of the phenolic compounds; this suggests a high content of free phenolic compounds in the full-fat oil-bearing plants. With the defatted meals more than 25%-30% of the total phenolic compounds were extracted only after alkaline and acid hydrolysis. Similar results were obtained by Varga and Diosady (1994) who reported the insoluble bound phenolic acids to be 26%-29% of total phenolic acids content in flaxseed meal, while the insoluble bound phenolic compounds were reported to be 5%-7% in different canola cultivars (Kozlowska *et al.,* 1975; Krygier *et al.,* 1982; Kozlowska *et al.,* 1983; Naczk and Shahidi, 1989). This suggests relatively high bound phenolic compounds in the defatted meal as compared to full-fat meal. These results are similar to those reported by Oomah and Mazza (1998) for flaxseed, rapeseed and canola meals (Blair and Reichert, 1984). Extraction without heat, solubilized 56% and 51% of the total phenolic compounds measured in full-fat soybean and flaxseed, respectively; methanol extraction with heat solubilized a further 34% and 37% of phenolic compounds from full-fat soybean and flaxseed, respectively. Dilute alkali and dilute acid solubilized the remaining 10%-12% of the total phenolic compounds that were not soluble in alcohol. The phenolic content in flaxseed was higher (20%-25%) than that found in soybean. For full-fat olive meal, extraction with and without heat solubilized 50%-60% and 30%-35% of the total phenolic compounds, respectively.

Table 3.2 shows total phenolic compounds content using sequential extraction of methanol extraction, methanol extraction/heat, alkaline hydrolysis and acid hydrolysis representing 4.69, 2.75 and 8.12 mg/g for full-fat and 3.60, 2.48 and 3.53 mg/g for defatted in flaxseed, soybean and olive meal, respectively. Oamah and Mazza (1998) reported that the content of phenolic compounds in full-fat and defatted flaxseed were 8.8 and 8.77 mg/g, respectively. Dabrowski and Sosulski (1984) reported that the total phenolic compounds content and esterified phenolic acids were 1.55 mg/g for dehulled, defatted flaxseed. Naczk *et al.* (1986) found that phenolic compounds content in soybean meal were 4.6 mg/g. Genovese *et al.* (2005) reported the phenolic compounds content in soybean was 1.4-2.4 mg/g, while Van Ruth *et al.* (2001) reported the phenolic compounds content in soybean was 2.5-4 mg/g. Caponio *et al.* (1999) reported that the total phenolic compounds content in olive meal were 0.175-0.272 mg/g and 0.387-0.455 mg/g in *Ogliurola salentina* and *Coratina* cultivars, respectively. Lesage-Gmeessen *et al.* (2001) found that the total phenolic compounds content was 0.2-0.25 mg/g using ethanol extraction, and 0.288-0.422 mg/g using ethyl acetate extraction in *Hojiblanca picual* cultivar. Caponio and Gomes (2001) reported the content of phenolic compounds in olive meal was 0.453 mg/g.

Table 3.1: Content of phenolic compounds from full-fat and defatted of flaxseed, soybean and olive meal using methanol and water as extractants.

Phenolic Compounds (mg/g as Gallic Acid)		
1. Extraction by water		
Sample	*Full-fat*	*Defatted*
Flaxseed	1.17±0.02	1.59±0.16
Soybean	1.87±0.12	3.05±0.15
Olive meal	3.23±0.35	9.78±0.08
1. Extraction with methanol after water extraction		
Sample	*Full-fat*	*Defatted*
Flaxseed	0.92±0.02	0.90±0.07
Soybean	0.45±0.03	1.60±0.01
Olive meal	1.25±0.04	1.12±0.05
2. Extraction by methanol		
Sample	*Full-fat*	*Defatted*
Flaxseed	1.90±0.07	1.20±0.02
Soybean	1.30±0.02	0.67±0.02
Olive meal	3.95±0.16	1.85±0.05
2. Extraction with water after methanol extraction		
Sample	*Full-fat*	*Defatted*
Flaxseed	1.61±0.1	0.67±0.06
Soybean	2.75±0.15	1.45±0.05
Olive meal	3.89±0.40	6.07±0.11

Results are means ± standard deviations of three determinations.

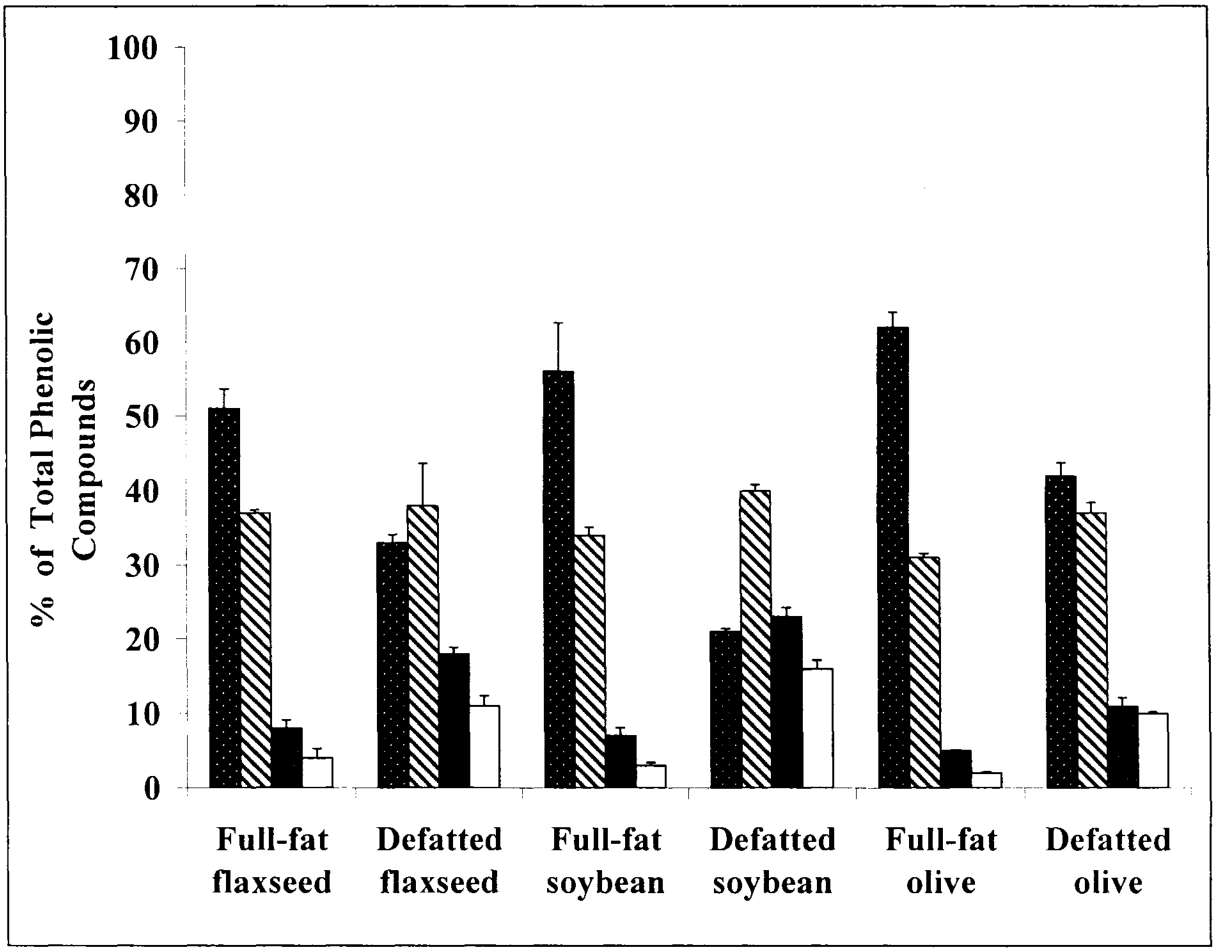


Figure 3.1: Phenolic compounds distribution in flaxseed, soybean and olive meal; ▩ Free phenolic compounds (methanol/23°C extraction); ▨ Free phenolic compounds (methanol/60°C extraction); ■ Bound phenolic compounds (NaOH hydrolysis); □ Bound phenolic compounds (HCl hydrolysis).

Table 3.2: Contents of free and bounds phenolic compounds from full-fat and defatted of flaxseed, soybean and olive meal as free and bound phenolic compounds.

Total Phenolic Compounds mg/g as Gallic Acid		
Extraction with methanol at 23°C (Free phenolic compounds)		
Sample	*Full-fat*	*Defatted*
Flaxseed	2.39±0.12	1.22±0.04
Soybean	1.54±0.18	0.51±0.01
Olive meal	5.00±0.17	1.48±0.06
Extraction with methanol at 60°C (Free phenolic compounds)		
Sample	*Full-fat*	*Defatted*
Flaxseed	1.73±0.02	1.33±0.20
Soybean	0.93±0.03	0.99±0.02
Olive meal	2.53±0.05	1.32±0.05
Extraction with methanol after hydrolysis with NaOH (Bound phenolic compounds)		
Sample	*Full-fat*	*Defatted*
Flaxseed	0.38±0.05	0.66±0.03
Soybean	0.19±0.03	0.59±0.03
Olive meal	0.43±0.01	0.37±0.04
Extraction with methanol after hydrolysis with HCl (Bound phenolic compounds)		
Sample	*Full-fat*	*Defatted*
Flaxseed	0.19±0.06	0.39±0.05
Soybean	0.09±0.01	0.39±0.03
Olive meal	0.16±0.01	0.36±0.01
Total phenolic compounds	*Full-fat*	*Defatted*
Flaxseed	4.69±0.25	3.60±0.32
Soybean	2.75±0.25	2.48±0.09
Olive meal	8.12±0.26	3.53±0.16

Results are means ± standard deviations of three determinations.

3.3.2 RP-HPLC and Mass Spectrometry Analysis of Phenolic Compounds

Figure 3.2 shows RP-HPLC chromatogram for mixture of standards of phenolic compounds. Figures 3.3 to 3.9 show the mass spectra of the standards of phenolic compounds to confirm their identification.

Figures 3.10, 3.11 and 3.12 show the chromatograms from extracts of defatted soybeans, flaxseed and olive meal, respectively. Table 3.3 shows the phenolic compounds for full-fat meals were similar to that obtained from defatted meals. The chromatograms for methanol extraction from flaxseed (Figure 3.11) showed gallic acid, protocatechuic acid and syringic acid as major peaks; the chromatograms for alkaline hydrolysis and acid hydrolysis after methanol/heat extraction (Figure 3.11) showed sinapic acid, ferulic acid and *p*-coumaric acid as major peaks. Figure 3.10 shows that free phenolic compounds identified in soybean include gallic acid, protocatechuic acid, hydroxybenzoic acid, syringic acid, sinapic acid, *p*-coumaric acid, ferulic acid, rutin, hesperidin and quercetin; while bound phenolic compounds include gallic acid, hydroxybenzoic acid, syringic acid and *p*-coumaric acid. Phenolic compounds previously identified in soybean meal include hydroxybenzoic acid, vanillic acid, protocatechuic acid, syringic acid, coumaric acid, ferulic acid, caffeic acid and sinapic acid (Klosterman *et al.*, 1955; Kozlowska *et al.*, 1983; Dabrowski and Sosulski, 1984; Kazimierz *et al.*, 1984; Westcott and Muir, 1996; Johnsson *et al.*, 2000), and gentistic, syringic acid, *o*-coumaric acid, *p*-coumaric and ferulic acid in soy flake (Arai *et al.* 1966; Maga and Lorenz, 1974; How and Morr, 1982). Arai *et al.* (1966) and Maga and Lorenz (1974) reported that syringic acid is the predominant phenolic compound in defatted soy flakes. While How and Morr (1982) reported that *o*-coumaric acid, *p*-coumaric acid and ferulic acid are predominant in defatted soy flake.

Figure 3.11 show chromatogram for free and bound phenolic compounds extracted from flaxseed: for free phenolic compounds include gallic acid, protocatechuic acid, hydroxybenzoic acid, syringic acid and *p*-coumaric acid, include bound phenolic compounds gallic acid, protocatechuic acid, hydroxybenzoic acid, syringic acid, *p*-coumaric acid, caffeic acid, sinapic acid, ferulic acid and cinnamic acid. Identification of hydroxybenzoic acid, vanillic acid, protocatechuic acid, syringic acid, coumaric acid, ferulic acid, caffeic acid and sinapic acid which exist as both free and bound forms has been reported in flaxseed (Klosterman *et al.*, 1955; Maga and Lorenz, 1974; Arai *et al.*, 1966; How and Morr, 1982; Kozlowska *et al.*, 1983; Dabrowski and Sosulski, 1984; Westcott and Muir, 1996; Johnsson *et al.*, 2000).

Figure 3.12 show the chromatograms of phenolic compounds in olive meal. Free phenolic compounds in olive meal include gallic acid, protocatechuic acid, hydroxybenzoic acid, vanillic acid, caffeic acid, syringic acid, sinapic acid, ferulic acid, *p*-coumaric acid, rutin, hesperidin, quercetin and cinnamic acid, and this is similar to phenolic compounds identified in olive meal and reported previously (Balice and Cera; 1984; Amiot *et al.*, 1986; Movsumov *et al.*, 1987; Mousa *et al.*, 1996; Poiana *et al.*, 1997; Servili *et al.*, 1999; Ryan *et al.*, 1999).

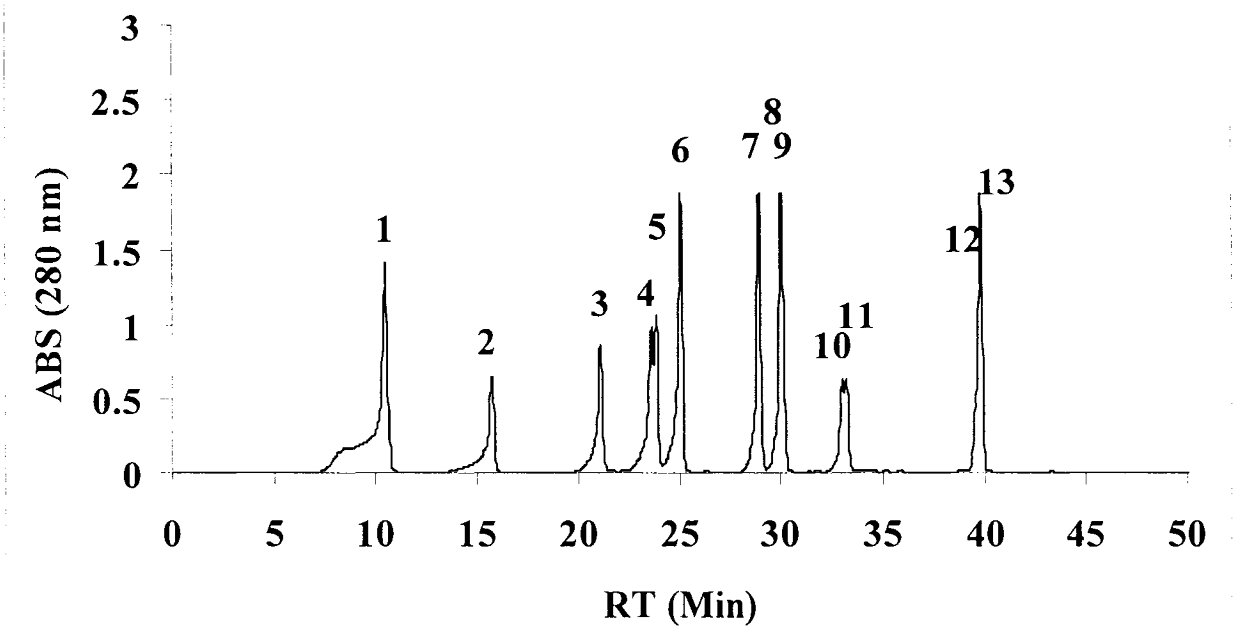


Figure 3.2: RP-HPLC separation of phenolic compounds for mixture of standards; Peaks: 1, Gallic acid; 2, Protocatechuic acid; 3, Hydroxybenzoic acid; 4, Vanillic acid; 5, Caffeic acid; 6, Syringic acid; 7, Sinapic acid; 8, Ferulic acid; 9, *p*-Coumaric acid; 10, Rutin; 11, Hesperidin; 12, Quercetin; 13, Cinnamic acid.

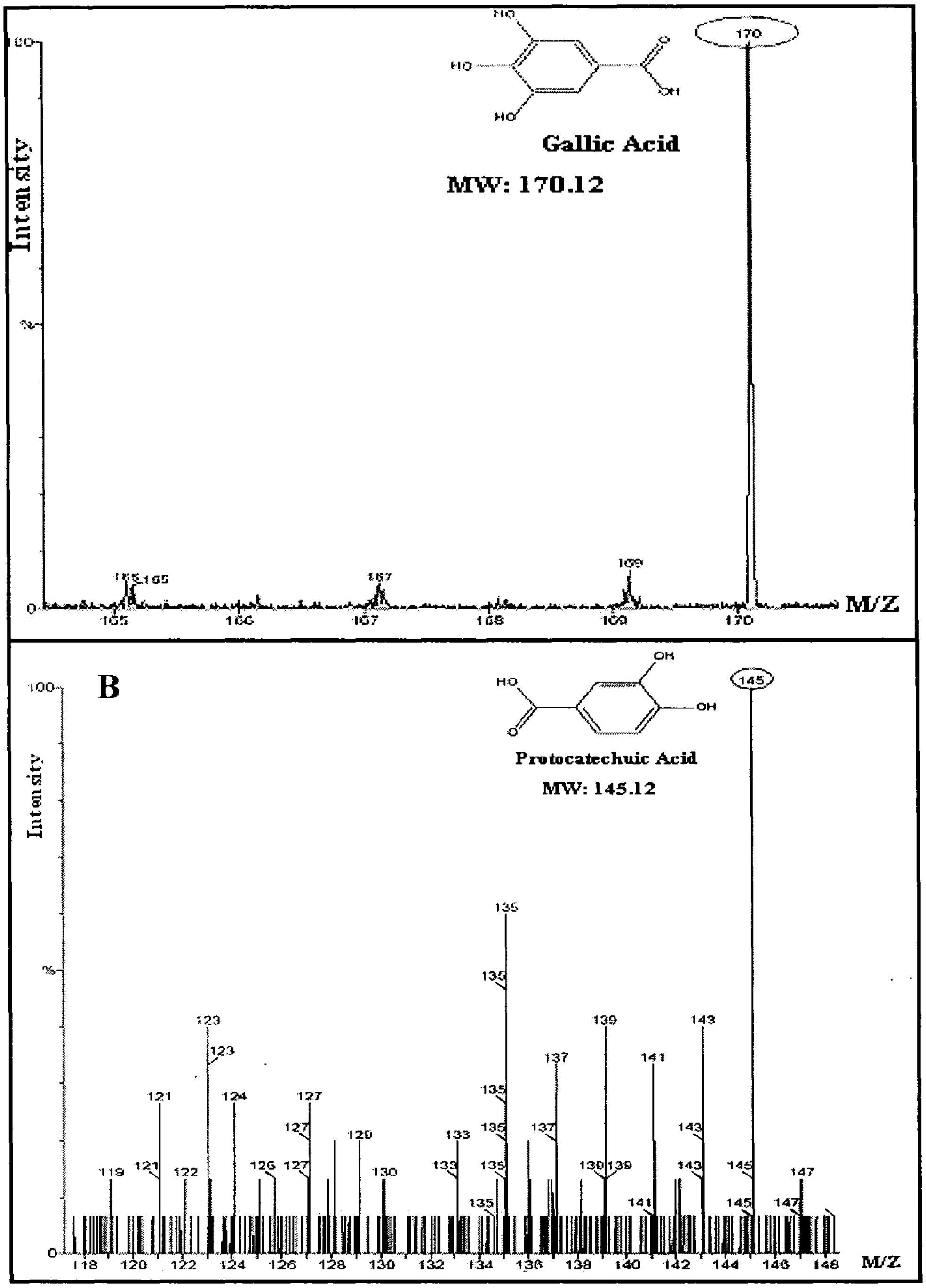


Figure 3.3: ESI/Mass Spectra of (A) Gallic acid; (B) Protocatechuic acid.

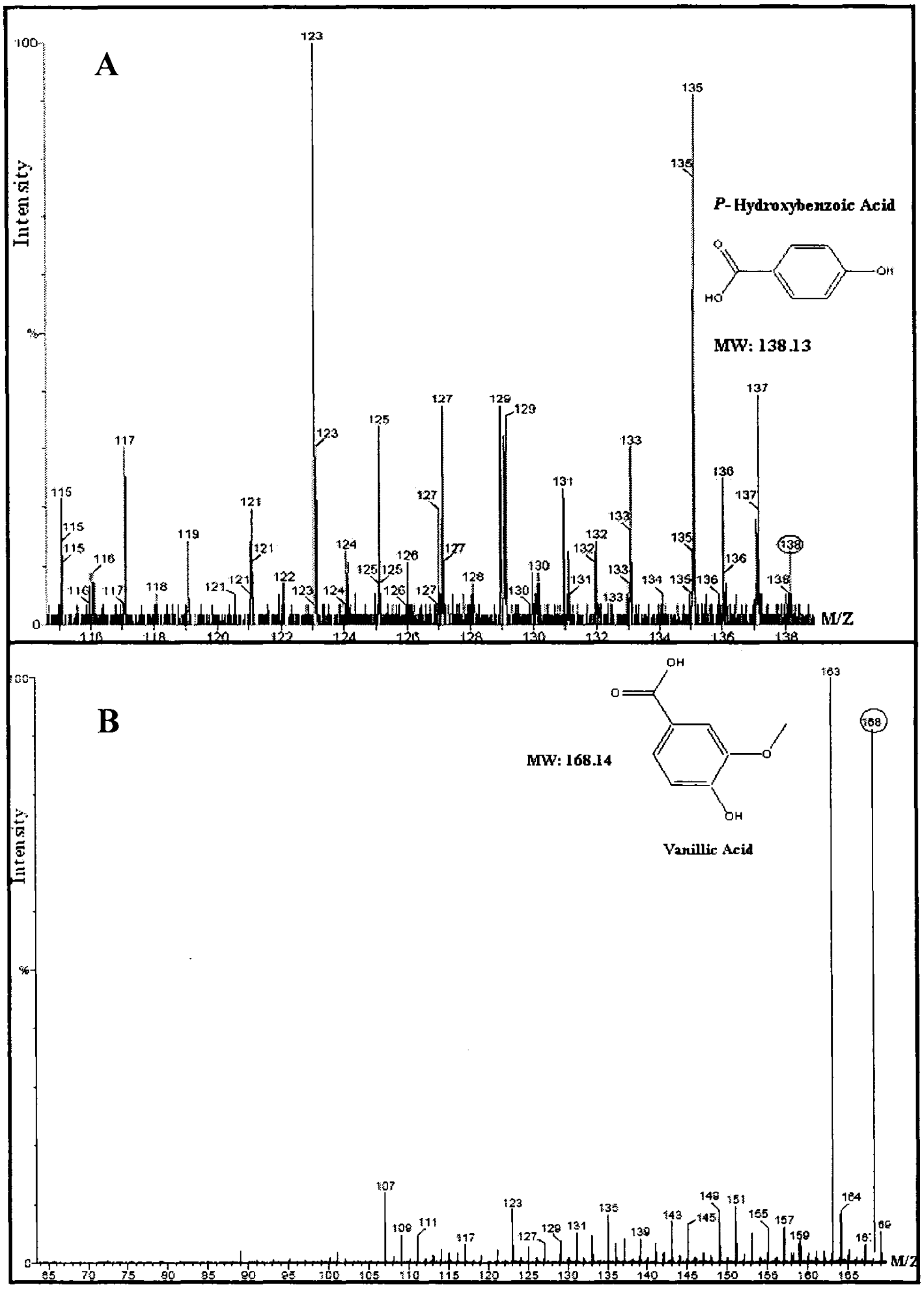


Figure 3.4: ESI/Mass Spectra of (A) *p*-Hydroxybenzoic acid; (B) Vanillic acid.

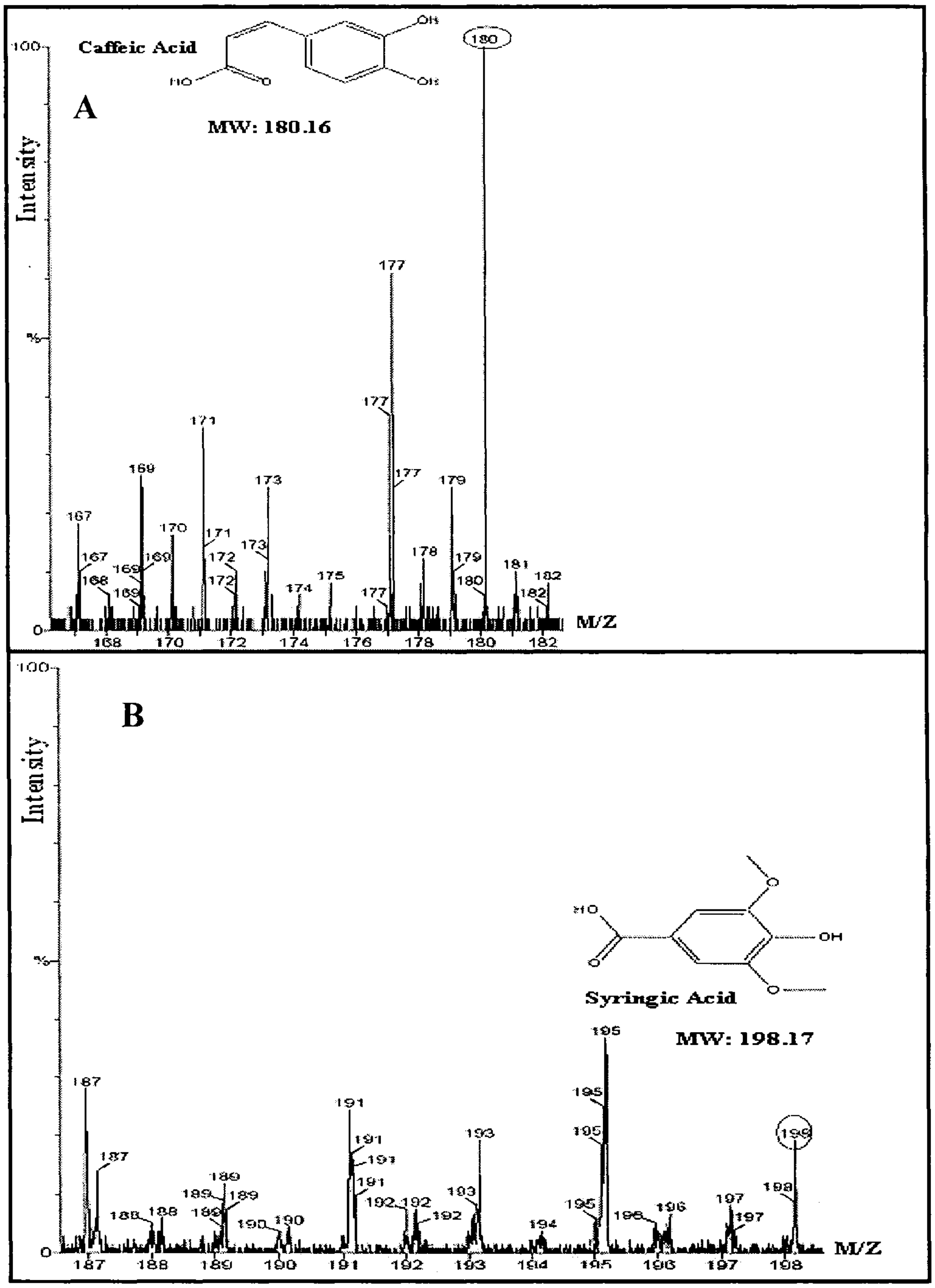


Figure 3.5: ESI/Mass Spectra of (A) Caffeic acid; (B) Syringic acid.

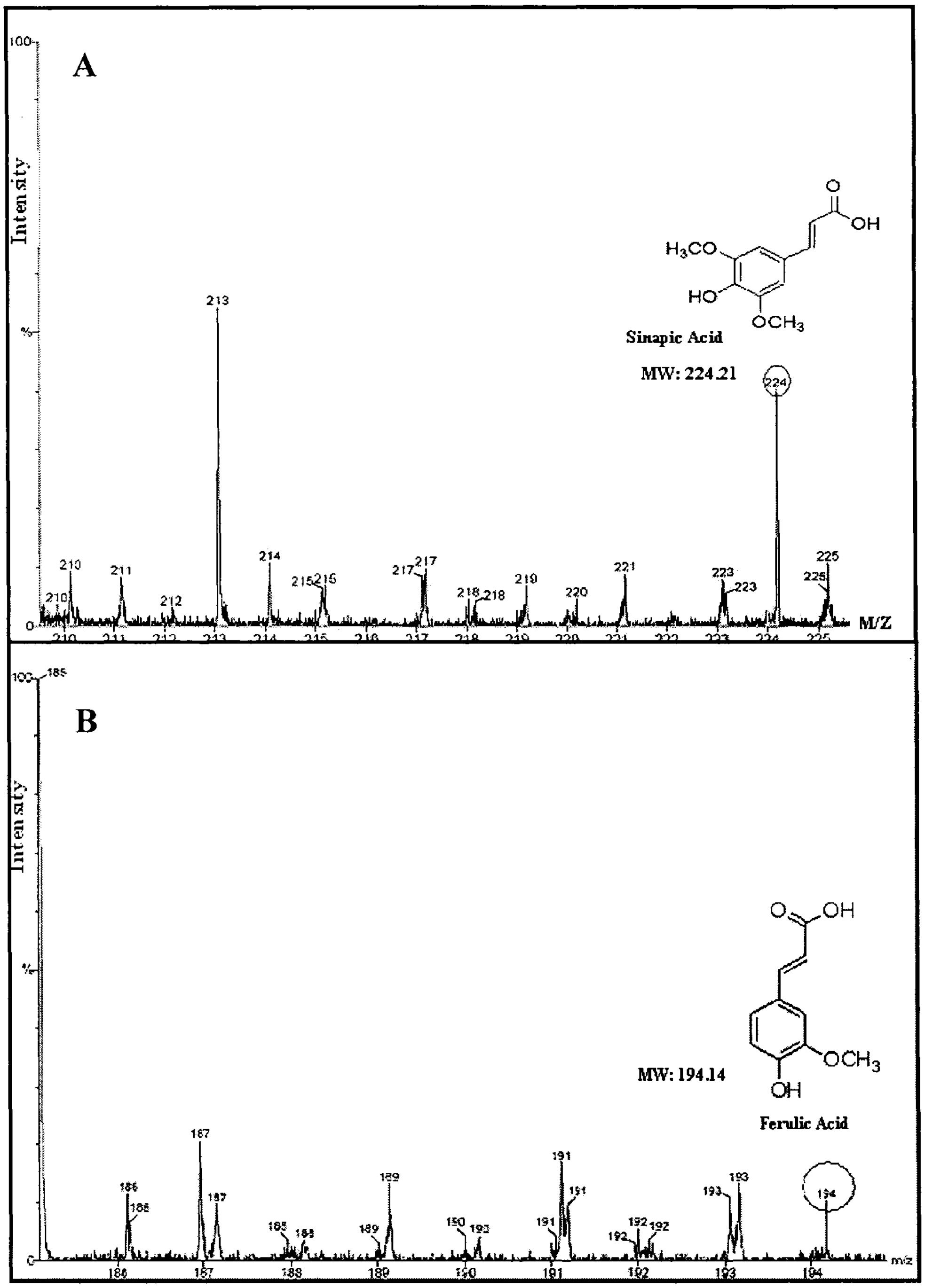


Figure 3.6: ESI/Mass Spectra of (A) Sinapic acid; (B) Ferulic acid.

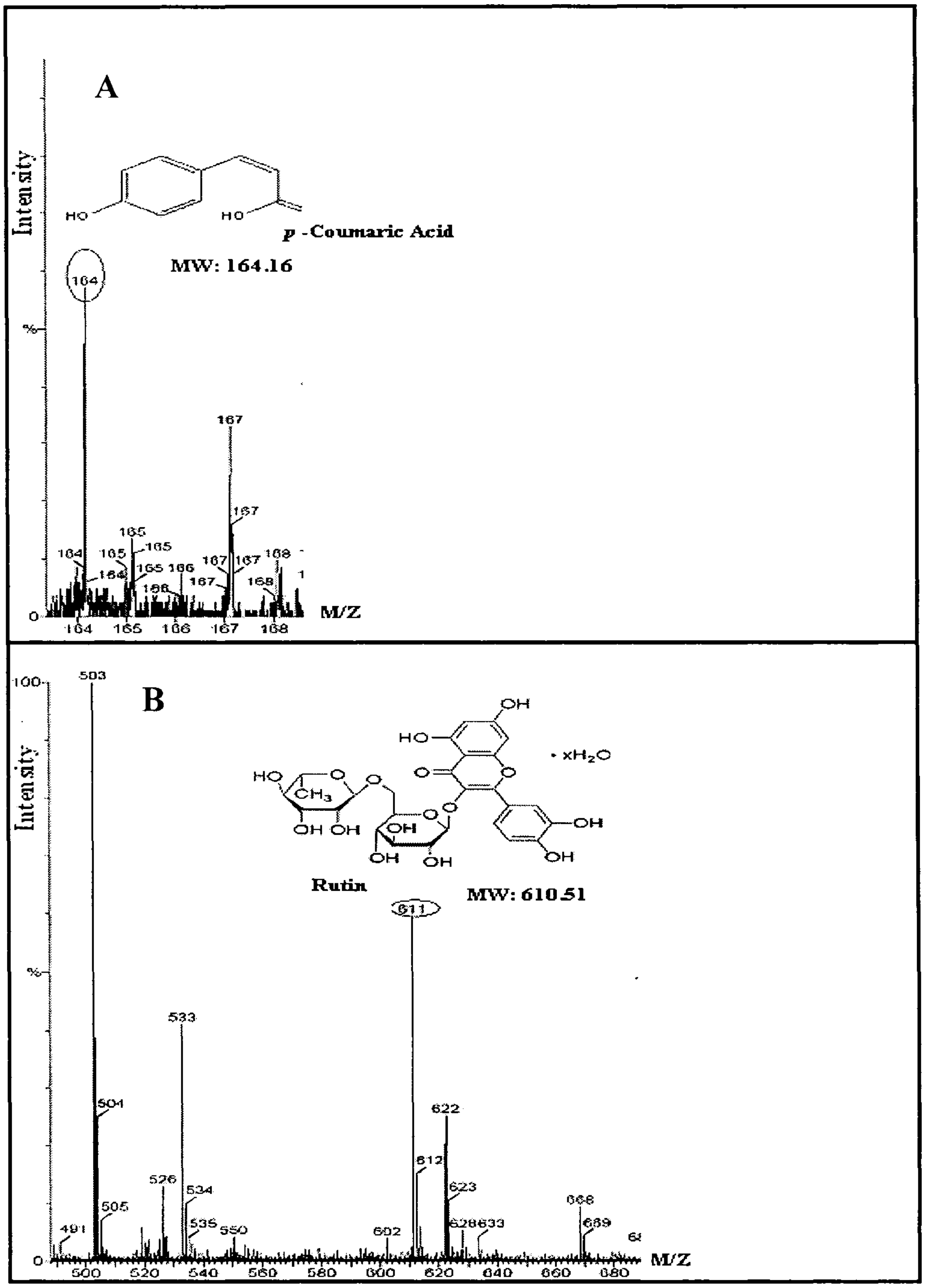


Figure 3.7: ESI/Mass Spectra of (A) *p*-Coumaric acid; (B) Rutin.

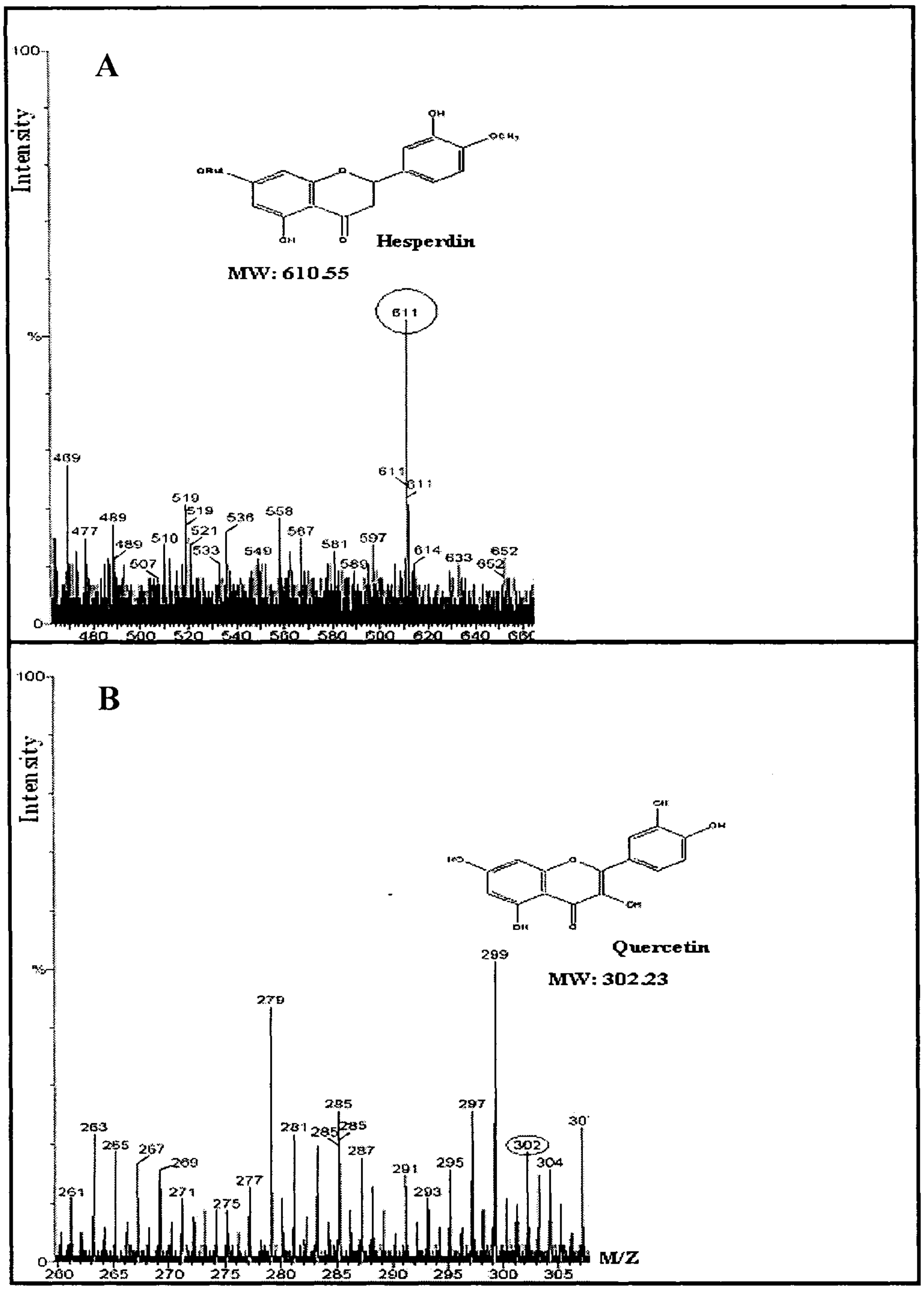


Figure 3.8: ESI/Mass Spectra of (A) Hesperidin; (B) Quercetin.

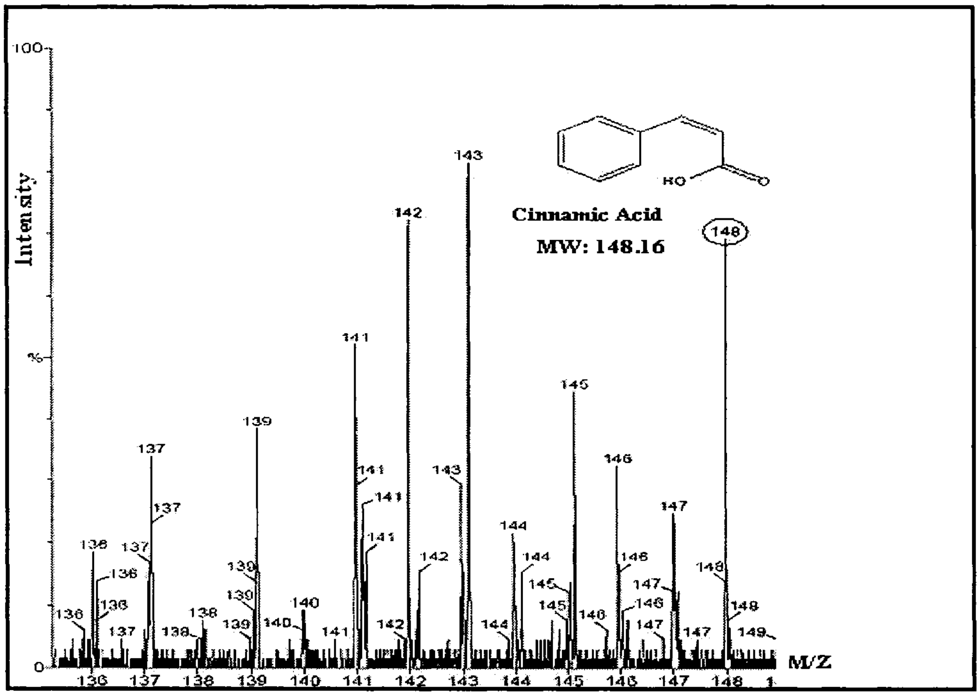


Figure 3.9: ESI/Mass Spectrum of cinnamic acid.

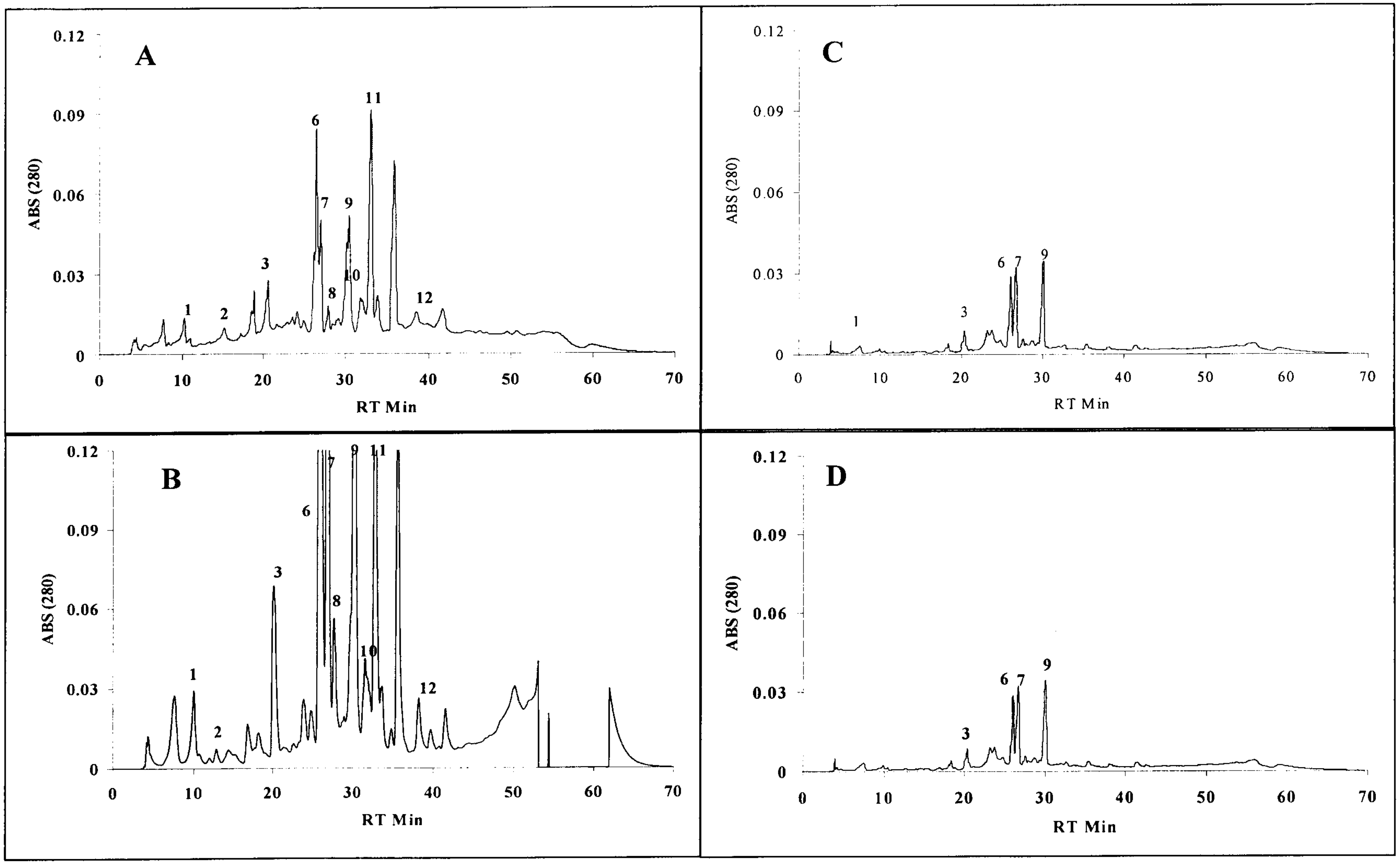


Figure 3.10: RP-HPLC separation of phenolic compounds for defatted soybean; (A) Free (extracted at room temperature); (B) Free (extracted at 60°C temperature); (C) Bound (base hydrolysis extraction); (D) Bound (acid hydrolysis extraction). Peaks: 1, Gallic acid; 2, Protocatechuic acid; 3, Hydroxybenzoic acid; 5, Caffeic acid; 6, Syringic aid; 7, Sinapic acid; 8, Ferulic acid acid; 9, *p*-Coumaric acid; 10, Rutin; 11, Hesperidin; 12, Quercetin.

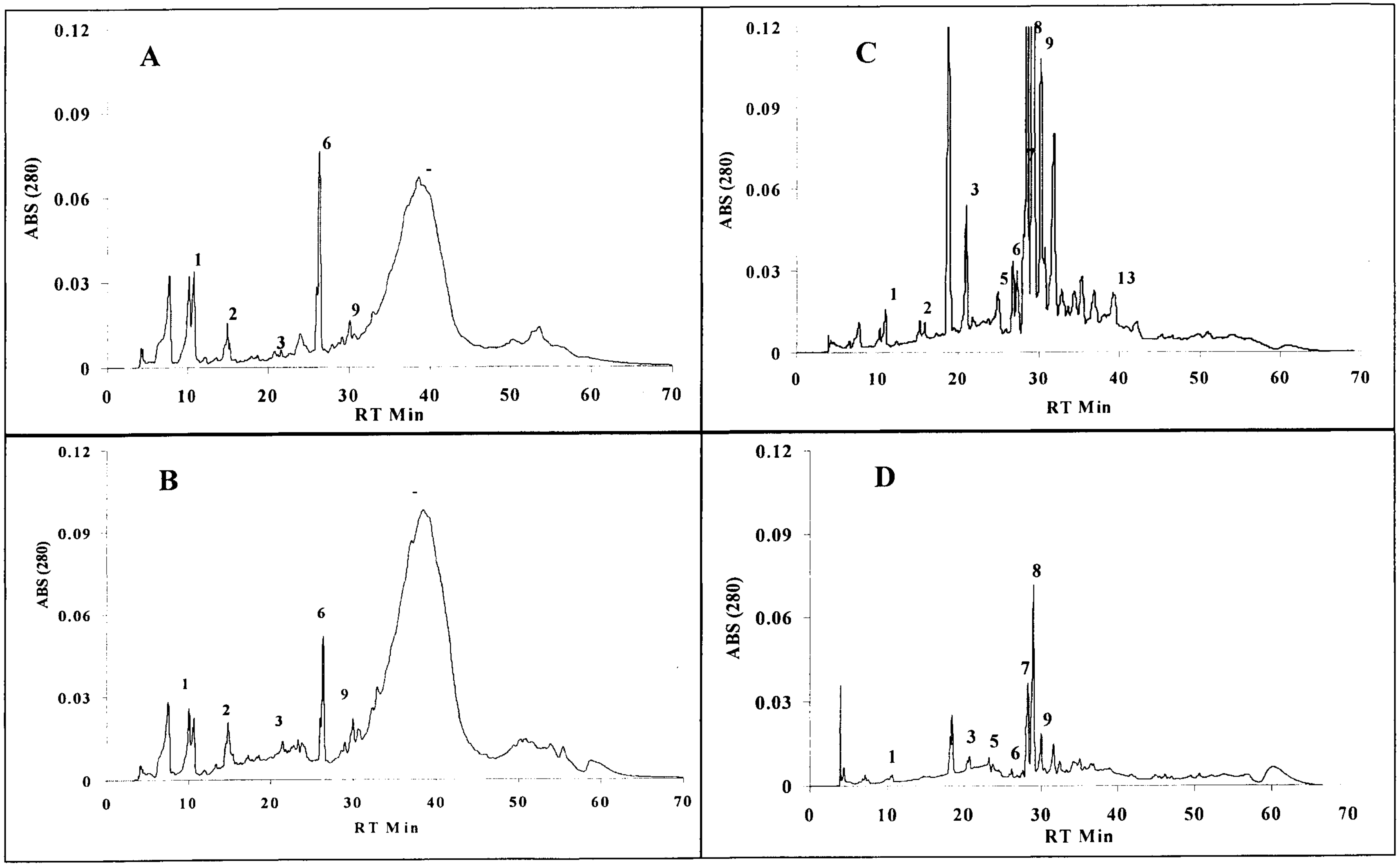


Figure 3.11: RP-HPLC separation of phenolic compounds for defatted flaxseed; (A) Free (extracted at room temperature); (B) Free (extracted at 60°C temperature); (C) Bound (base hydrolysis extraction); (D) Bound (acid hydrolysis extraction). Peaks: 1, Gallic acid; 2, Protocatechuic acid; 3, Hydroxybenzoic acid; 5, Caffeic acid; 6, Syringic acid; 7, Sinapic acid; 8, Ferulic acid; 9, *p*-Coumaric acid; 13, Cinnamic acid.

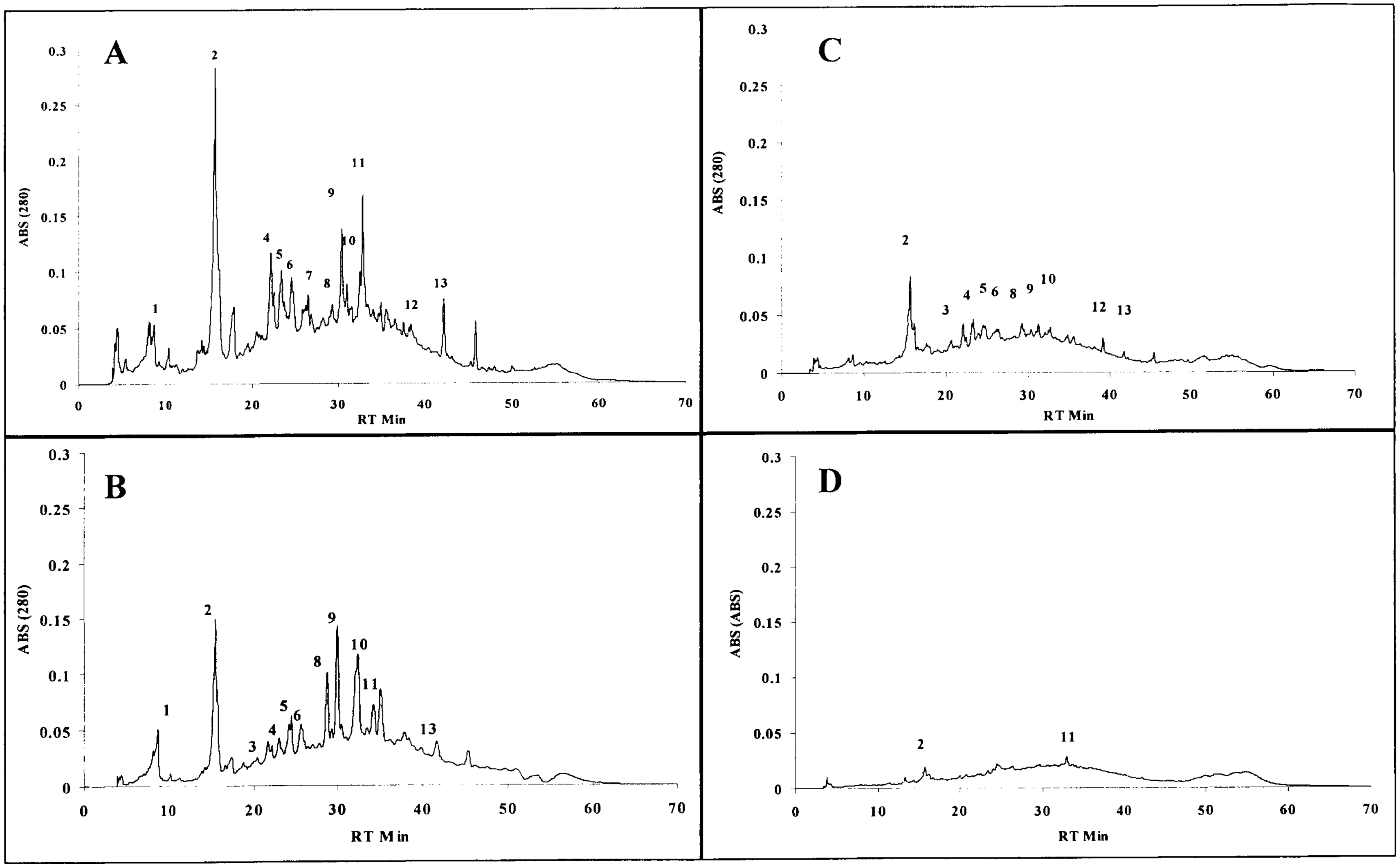


Figure 3.12: RP-HPLC separation of phenolic compounds for defatted olive; (A) Free (extracted at room temperature); (B) Free (extracted at 60°C temperature); (C) Bound (base hydrolysis extraction); (D) Bound (acid hydrolysis extraction). Peaks: 1, Gallic acid; 2, Protocatechuic acid; 3, Hydroxybenzoic acid; 4, Vanillic acid; 5, Caffeic acid; 6, Syringic acid; 7, Sinapic acid; 8, Ferulic acid; 9, *p*-Coumaric acid; 10, Rutin; 11, Hesperidin; 12, Quercetin; 13, Cinnamic acid.

Table 3.3: Identifications of phenolic compounds obtained using different extractions techniques (RP-HPLC and ESI-MS) analysis in flaxseed, soybean and olive.

Peak #	Phenolic Compounds	Literature[a]	Flaxseed		Literature[b]	Soybean		Literature[C]	Olive	
			HPLC	MS		HPLC	MS		HPLC	MS
1	Gallic Acid	-	X	X	X	X	X	X	X	X
2	Protocatechuic Acid	X	X	X	X	X	X	X	X	X
3	Hydroxybenzoic Acid	X	X	X	X	X	X	X	X	X
4	Vanillic Acid	X	-	-	X	-	-	X	X	X
5	Caffeic Acid	X	X	X	X	X	X	X	X	X
6	Syringic Acid	X	X	X	X	X	X	X	X	X
7	Sinapic Acid	X	X	X	X	X	X	X	X	X
8	Ferulic Acid	X	X	X	X	X	X	X	X	X
9	*P*-Coumaric Acid	X	X	X	X	X	X	X	X	X
10	Rutin	-	-	-	X	X	X	X	X	X
11	Hesperidin	-	-	-	X	X	X	X	X	X
12	Quercetin	-	-	-	X	X	X	X	X	X
13	Cinnamic Acid	-	X	X	X	-	-	X	X	X

X; Represent the presence of phenolic compounds; (-): Represent not identified.

(a): Klosterman *et al.* (1955); Westcott and Muir (1996); Johnsson *et al.* (2000); Kozlowska *et al.* (1983); Dabrowski and Sosulski (1984); Sosulski (1984); Maga and Lorenz (1974); Arai *et al.* (1966); How and Morr (1982). (b): Maga and Lorenz (1974); Dabrowski and Sosulski (1984); How and Morr (1982); Klosterman *et al.* (1955); Westcott and Muir (1996); Johnsson *et al.* (2000); Arai *et al.* (1966); Hammerschmidt and Pratt (1978); Pratt and Birac (1979). (c): Mousa *et al.* (1996); Montedoro *et al.* (1992); Akasbi *et al.* (1993); Tsimidou (1998); Baldi *et al.* (1995); Vlahov (1992).

3.3.3 Antioxidant Activity of Phenolic Extracts

Table 3.4 and Figure 3.13 show the antioxidant activity of free and bound phenolic compounds for full-fat and defatted flaxseed, soybean, olive meal based on methanol extraction, methanol extraction/heat, alkaline hydrolysis and acid hydrolysis carried out in sequence. The full-fat meals showed higher antioxidant activity as compared to defatted meals. Bound phenolic compounds that were extracted using alkaline hydrolysis showed higher antioxidant activity 68% and 25% from both full-fat and defatted soybean meals, respectively compared to free phenolic compounds. The free phenolic compounds extracted from full-fat flaxseed had higher antioxidant activity (58%) compared to bound phenolic compounds extracted from full-fat flaxseed (7%-21%). There are many methods for determination of antioxidant activity and every method has limitations (Gorinstein *et al.*, 2001; Yu *et al.*, 2002; Ou *et al.*, 2002). Gorinstein *et al.* (2003) reported that the best method for determination of the antioxidant activity is the β-carotein/linoleic acid assay. Yu *et al.* (2002) reported that there is no correlation between total phenolic compounds content and antioxidant activity. Kahkonen *et al.* (1999) reported that phenolic extract gave 35% inhibition for flaxseed using methyl linoleate oxidation assay and 61% using the β-carotein/linoleice acid assay for full-fat flaxseed (Velioglu *et al.*, 1998). The antioxidant activity for soybean was 6%-62% for 2, 2-Diphenyl picryl hydrazyl (DPPH) assay and 2-Thiobarbituric acid (TBA) assay (Lee *et al.*, 2002). Velioglu *et al.* (1998) found there is a relationship between the total phenolic compounds and AA% in flaxseed. Huang *et al.* (2003) found that the AA% for phenolic compounds extracted from tofu and soybean were 48%-54% and 50%-55%, respectively using DPPH assay. Kania *et al.* (2004) reported there is no correlation between the AA% and total phenolic compounds content in soybean. AA%

for phenolic compounds from soybean using β-carotein/linoleic acid was found 38%-49% (Genovese *et al.*, 2005). Valavanidis *et al.* (2004) reported that for soybean oil extracted with methanol needs 45-48 mg/ml of phenolic compound to inhibite 50% of DPPH. 68% antioxidant activity of soybean extract had been reported using DPPH assay (Georgetti *et al.*, 2006). Lee *et al.* (2000) reported that the water extract from soybean inhibit 95% of lipid oxidation using lipid/ malonaldehyde (MA) assay. Romero *et al.* (2004) found that the AA% for water extract from soybean was 57%-80% using DPPH assay and 30%-92% using linoleic acid/β-carotene assay.

Free phenolic compound extracted from full-fat olive meal showed higher antioxidant activity (67% and 58%) as compared to defatted meal (10% and 48%) using methanol at room temperature and methanol/heat, respectively. The bound phenolic compounds extracted after base hydrolysis from full-fat olive meal gave higher antioxidant activity (47%) compared to defatted meal (9%), while for the bound phenolic compounds extracted after acid hydrolysis defatted meal showed a higher antioxidant activity (48%) in comparison to full-fat meal (34%). Mancini-Filho *et al.* (2005) reported that the antioxidant activity for olive meal using β-carotein/linoleic assay was 61%. AA% for olive oil extracted using methanol was 22-24 mg/ml of phenolic compound to inhibit 50% of DPPH (Valavanidis *et al.*, 2004). Papadopoulos *et al.* (2003) reported that the 37% as antioxidant activity were measured using chemluminescence measurement in olive oil. The antioxidant activity found in olive oil was different using two different methods: 10%-23% for DPPH assay and 13%-33% using β-carotene/linoleate assay; Litridou Maria *et al.* (1997) reported that the AA% as reactive oxygen species was 98% for oil of olive seed and 65%-45% for virgin olive oil.

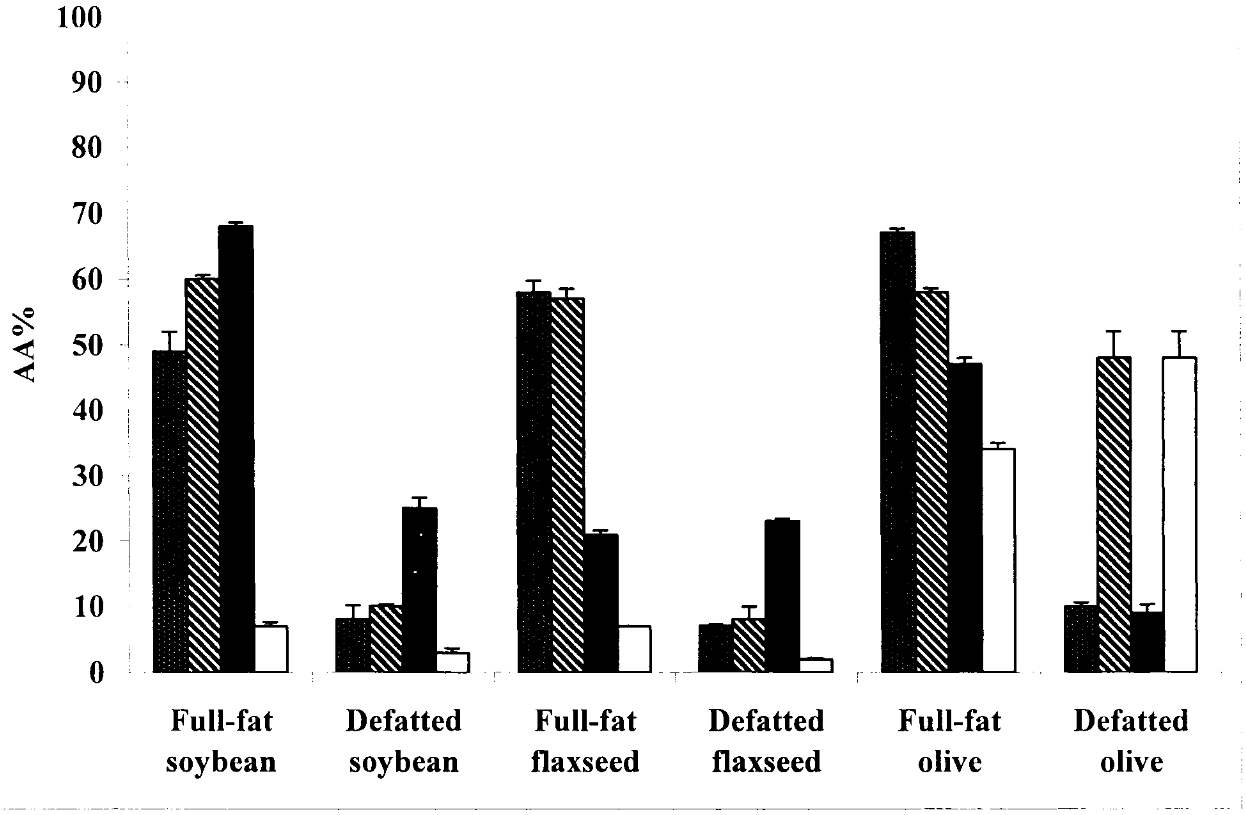


Figure 3.13: Antioxidant Activity (AA%) of flaxseed, soybean and olive meal; ▩ Free phenolic compounds (methanol/23°C extraction);▨ Free phenolic compounds (methanol/60°C extraction);■ Bound phenolic compounds (NaOH hydrolysis); □ Bound phenolic compounds (HCl hydrolysis).

Table 3.4: Determination of antioxidant activity (AA%) of free and bound phenolic compounds extracted from full-fat and defatted flaxseed, soybean olive meal.

Mode of Extraction	Full-fat flaxseed	Defatted flaxseed	Full-fat soybean	Defatted soybean	Full-fat olive meal	Defatted olive meal
Free phenolic compounds extracted at room temperature	58±1.7	7±0.6	49±3.0	8±2.1	67±0.6	10±0.6
Free phenolic compounds extracted at 60°C temperature	57±1.5	8±0.7	60±0.6	10±0.3	58±0.6	48±4.0
Bound phenolic compounds extracted after base hydrolysis	21±0.6	23±0.0	68±0.6	25±1.6	47±1.0	9±1.3
Bound phenolic compounds extracted after acid hydrolysis	7±0.0	2±0.2	7±0.6	3±0.7	34±1.0	48±4.0

Results are means ± standard deviation of four determinations.

CHAPTER 4

EFFECT OF REMOVAL OF PHENOLIC COMPOUNDS ON MOLECULAR CHARACTERISTICS OF SOYBEAN AND FLAXSEED PROTEINS

4.1 Justification

In Chapter 3, the identification of phenolic compounds in soybean, flaxseed and olive meal and antioxidant activity of the phenolic compounds were reported. In this chapter similar investigations were carried out on proteins isolated from soybean and flaxseed; olive was not used since it is not commonly used as a source of food proteins. Proteins were isolated from full-fat and defatted soybean and flaxseed using sodium hydroxide extraction and isoelectric precipitation, followed by extraction of free and bound phenolic compounds. The effects of removal of the phenolic compounds on molecular characteristics of the protein isolates were studied using RP-HPLC, polyacrylamide gel electrophoresis (SDS-PAGE and Native-PAGE) and electron spray ionization-mass spectrometry (ESI-MS).

4.2 Materials and Methods

4.2.1 Materials

Full-fat and defatted soybean and flaxseed described in Section 3.2.1 were used.

4.2.2 Preparation of Protein Isolates from Oil-seeds

Isoelectric precipitation for flaxseed and soybean proteins was carried out according to the method described by Dev and Quensel (1986) and Krase *et al.* (2002) with some modifications. Samples (10 g) of full-fat (F) and defatted (D) soybean (s) and flaxseed (f) were mixed with dilute NaOH (2.0 N, 100 ml). The mixture was adjusted to pH 11.0 and stirred (23°C/60 min), then centrifuged (10,000 X g, 30 min). The extract was filtered through cheese cloth and the residue was discarded. The extract was adjusted to pH 4.6 using dilute HCl (0.1 N). The precipitated protein isolate was separated by centrifugation (10,000 X g, 15 min), then freeze dried. The protein isolate obtained by this technique was designated as (I).

4.2.3 Extraction of 'Free' Phenolic Compounds

Samples (1 g) of protein isolate were extracted as described in Section 3.2.2.3. Total phenolic content was determined in extract. The residue after extraction of free phenolic compounds was divided into two parts; one part was used for further phenolic analysis as described in Section 4.2.4 (bound phenolic compound extraction), the total phenolic content was determined in extract, while the second part was kept for protein analysis (molecular characterization).

4.2.4 Extraction of 'Bound' Phenolic Compounds

The residue remaining from Section 4.2.3 was subjected to basic and acidic hydrolysis followed by methanol extraction according to the method described in Section 3.2.2.4. Total phenolic contents were determined in extracts. The residue was kept for further protein analysis.

4.2.5 Determination of Total Phenolic Content

The procedure to quantify total phenolic content of the extracts was similar to that described in Section 3.2.3.

4.2.6 RP-HPLC Analysis of Phenolic Compounds

The procedures for RP-HPLC preparation and separation of phenolic compounds were similar to that described in Section 3.2.4.

4.2.7 Determination of the Antioxidant Activity

The procedures to measure antioxidant activity of phenolic extracts were similar to that described in Section 3.2.6.

4.2.8 Polyacrylamide Gel Electrophoresis

4.2.8.1 Native-PAGE

Polyacrylamide gel electrophoresis (PAGE) of protein isolates obtained from Sections 4.2.2, 4.2.3 and 4.2.4 was performed under native conditions according to the method described by Davis (1964), using a mini-protean II electrophoresis cell unit. Sample solutions (25 μl) of freeze-dried proteins precipitate were dissolved in sample buffer (1.5 M Tris-HCl pH 8.8, 2% glycerol and 0.1% bromophenol blue). Electrophoresis migration was performed for 2-3 h at constant current (10 mA/gel) in 10% running buffer using Tris-glycine buffer pH 8.3. Gels were stained with Coomassie Brilliant Blue R-250 (0.1% W/V in 70% water, 20% methanol, 10% acetic acid) and destained with the same solvent system (but without dye).

4.2.8.2 SDS-PAGE

SDS-PAGE electrophoresis for protein isolates obtained from Sections 4.2.2, 4.2.3 and 4.2.4 was carried out on using the technique described by Laemmli (1970). Sample solutions (25 μl) of freeze-dried proteins precipitate were dissolved in sample buffer (10% SDS, 0.5 M β-mercaptoethanol, 0.5 M Tris-HCl pH 6.8, 2% glycerol and 0.1% bromophenol blue), then heated at 95°C for 5-10 min. Electrophoresis was performed at constant current 15 mA/gel for 1.5-2 h. The protein sub-units bands was stained with 0.1% Coomassie Brilliant Blue R-250 in 70% water, 20% methanol and 10% acetic acid, and destained in the same solvent system (but without dye).

4.2.9 RP-HPLC Analysis of Isolated Proteins

Protein isolates from Sections 4.2.2, 4.2.3 and 4.2.4 was dissolved in 0.1% trifluoroacetic acid (TFA) at a concentration of 40 mg/ml and centrifugated (5,000 X g, 10 min). The supernatant was filtered through (0.45 μm Millipore^{TM} membrane filters) and subjected to RP-HPLC analysis. HPLC analysis was carried out using the method described by El-Ramahi (2003) with a Beckman (CA, USA) liquid chromatography equipped with a

Programmable Solvent Module (model 126) for high-pressure solvent delivery, a Beckman manual injector with a 100 μl loop and a Programmable Detector Module (Model 166). Spectral and chromatographic data were analyzed by the Gold System (version V810), translated into PRN format for Microsoft Excel manipulation. For chromatographic separation, 100 μl sample was injected into C_{18} reversed phase column (5 micron, 250 X 4.6 mm, J.T Baker Inc., US). The sample was eluted at a flow rate of 1 ml/ min with following two-buffer gradient system: solvent A, 0.1% TFA in water (V/V); solvent B, 30% acidified water (0.1% TFA) in acetonitrile, starting at 10% solvent B for 10 min, and increasing linearly from 10% to 90% over 30 min. The initial conditions were then re-established over 10 min. The UV detector was calibrated and the eluate was monitored at 210 nm. Fractions eluating from the column were collected, pooled and concentrated, and freeze-dried then analyzed by SDS-PAGE, Native-PAGE and ESI-MS.

4.2.10 Electrospray Ionization Mass Spectrometry (ESI-MS)

To determine the molecular weight of protein fractions separated by RP-HPLC (Section 4.2.9) were dissolved in 10% acetic acid. The sample extensively mixed by vortexing followed by centrifugation at (5,000 X g, 10 min), the supernatant was then filtered through 0.45 μm Millipore^{TM} membrane and subjected to ESI-MS. The procedure for analysis for method of preparation of protein was similar to that described in Section 3.2.8.

4.3 Results and Discussion

4.3.1 Phenolic Compounds Distribution

Table 4.1 and Figure 4.1 show the distribution of phenolic compounds in protein isolates (I) from full-fat (F) and defatted (D) flaxseed (f) and soybean (s) based on sequential extractions; with methanol extraction, methanol extraction/heat, alkaline hydrolysis and acid hydrolysis. Generally, the total phenolic contents for protein isolates from defatted meals were higher as compared to protein isolates from full-fat meals. The total content of phenolic compounds using the four modes of extractions measured 5.09, 1.96, 7.11 and 3.42 mg/g for FfI, FsI, DfI and DsI, respectively. The free phenolic compounds (extracted with no prior hydrolysis) ranged from 70%-90% in the protein isolates from full-fat and defatted flaxseed and soybean meals. The remaining 10%-30% was considered as bound phenolic compounds. For flaxseed protein isolate a higher quantity of both free and bound phenolic compounds was extracted when compared with protein isolate from soybean. Protein isolates from full-fat soybean showed higher content of bound phenolic compounds (20%) compared to protein isolates from defatted soybean (11%). Based on our literature search, the phenolic compound distributions in protein isolates from these oilseeds have not been reported previously in the literature.

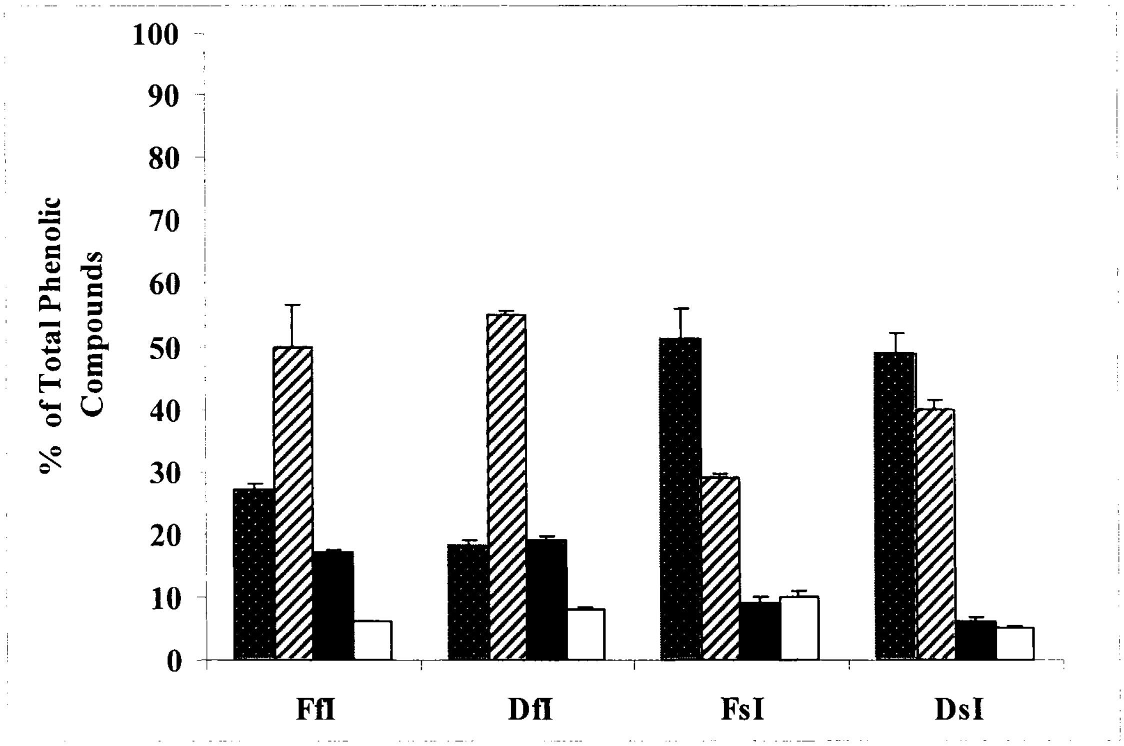


Figure 4.1: Phenolic compounds distribution of protein isolate (I) from soybean (s) and flaxseed; ▦ Free phenolic compounds (methanol/23°C extraction); ▨ Free phenolic compounds (methanol/60°C extraction); ■ Bound phenolic compounds (NaOH hydrolysis); □ Bound phenolic compounds (HCl hydrolysis).

Table 4.1: Content of total free and total bound phenolic compounds for protein isolates (I) from full-fat (F) and defatted (D) soybean (s) and flaxseed (f).

Total Phenolic Compounds (mg/g as Gallic Acid)		
Extraction with methanol at 23°C (Free phenolic compounds)		
Sample	*Full-fat*	*Defatted*
fI	2.35±0.05	1.27±0.08
sI	1.00±0.10	1.70±0.10
Extraction with methanol at 60°C (Free phenolic compounds)		
Sample	*Full-fat*	*Defatted*
fI	2.04±0.33	3.93±0.04
sI	0.57±0.01	1.35±0.05
Extraction with methanol after hydrolysis with NaOH (Bound phenolic compounds)		
Sample	*Full-fat*	*Defatted*
fI	0.41±0.02	1.32±0.04
sI	0.18±0.02	0.19±0.02
Extraction with methanol after hydrolysis with HCl (Bound phenolic compounds)		
Sample	*Full-fat*	*Defatted*
fI	0.29±0.01	0.59±0.02
sI	0.21±0.02	0.18±0.02
Total phenolic compounds	*Full-fat*	*Defatted*
fI	5.09±0.41	7.11±0.18
sI	1.96±0.15	3.42±0.19

Results are means ± standard deviations of three determinations.

4.3.2 HPLC Analysis of Phenolic Compounds

Figure 4.2 and Table 4.2 show the phenolic compounds identified in protein isolates from soybean. The identification of free and bound phenolic compounds in protein isolate from full-fat soybean was similar to phenolic compounds in protein isolate from defatted soybean. The free phenolic compounds include gallic acid, hydroxybenzoic acid, caffeic acid, syringic acid, sinapic acid, ferulic acid, *p*-coumaric acid, hesperidin and quercetin. Bound phenolic compounds identified after basic hydrolysis were *p*-coumaric acid, syringic acid and hesperidin. How and Morr (1982) identified the free phenolic acids genistic, syringic, *o*-coumaric, *p*-coumaric and ferulic acid in soy protein isolate.

Figure 4.3 and Table 4.2 show phenolic compounds profiles identified in protein isolates from defatted flaxseed; the chromatogram of free phenolic compounds for protein isolate from full-fat flaxseed was similar to that obtained with protein isolate from defatted flaxseed. The free phenolic compounds identified include gallic acid, protocatechuic acid, hydroxybenzoic acid, caffeic acid, syringic acid, ferulic acid and *p*-coumaric acid. The bound phenolic compounds identified include gallic acid, hydroxybenzoic acid, syringic acid, caffeic acid, ferulic acid and *p*-coumaric acid; these phenolic compounds exist in nature in both free and bound forms with protein. A literature, there is search revealed no information on the identification of free and bound phenolic compounds in protein isolates from flaxseed and soybean. Figure 4.3A2 shows chromatograms of hydrolyzed fraction X; the identified phenolic compounds were hydroxybenzoic acid, ferulic acid and *p*-coumaric acid; this suggests that these three phenolic compounds had an interaction with protein.

Figure 4.4 and Table 4.2 show profiles for bound phenolic compounds identified in protein isolates from defatted and full-fat flaxseed; bound phenolic compounds identified for protein isolate from defatted were slightly similar to protein isolate from full-fat flaxseed except the presence of sinapic acid. In general, these results indicate that protein-phenolic interactions occur in protein isolates from both soybean and flaxseed.

4.3.3 Antioxidant Activity of Phenolic Extracts in Protein Isolates

Table 4.3 and Figure 4.5 show the antioxidant activity of phenolic extracts for protein isolates from full-fat and defatted flaxseed and soybean. Generally, protein isolates from full-fat flaxseed and soybean meals showed a higher antioxidant activity as compared to protein isolates from defatted flaxseed and soybean meals, The bound phenolic compounds extracted from protein isolate of defatted flaxseed after alkaline and acid hydrolysis showed 15% and 32% antioxidant activity, respectively; no antioxidant activity was observed for bound phenolic compounds in protein isolates from defatted soybean. For protein isolate from full-fat meals, the bound phenolic compounds extracted after alkaline and acid hydrolysis had antioxidant activity 30% and 35% for flaxseed, respectively, and 32% and 16% for soybean, respectively.

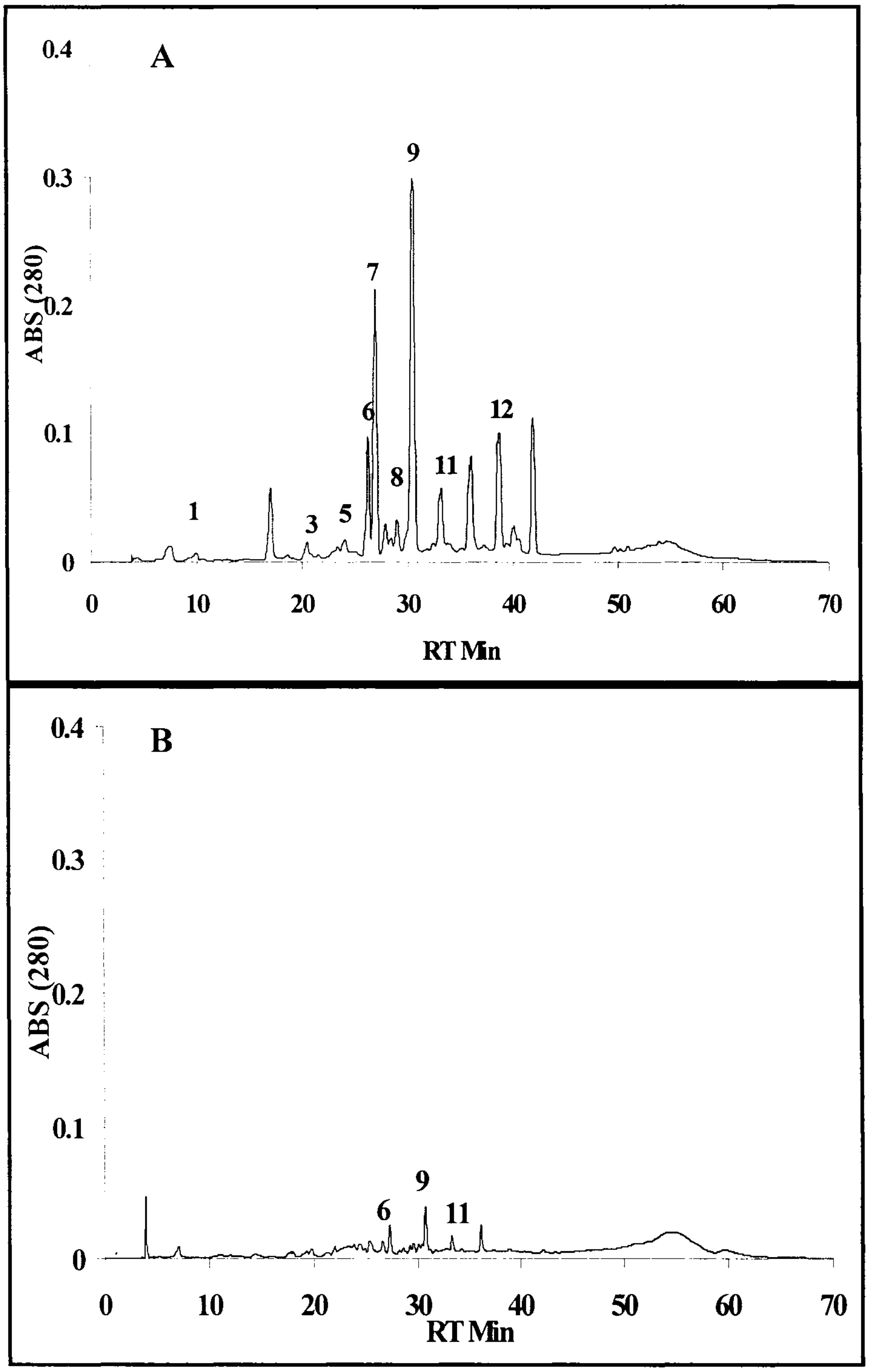


Figure 4.2: RP-HPLC of phenolic compounds for defatted protein isolate from soybean; (A) Free phenolic compounds; (B) Bound phenolic compounds extracted after base hydrolysis; Peaks: 1; Gallic acid; 3, Hydroxybenzoic acid; 5, Caffeic acid; 6, Syringic acid; 7, Sinapic acid; 8, Ferulic acid; 9, *p*-Coumaric acid; 11, Hesperidin; 12, Quercetin.

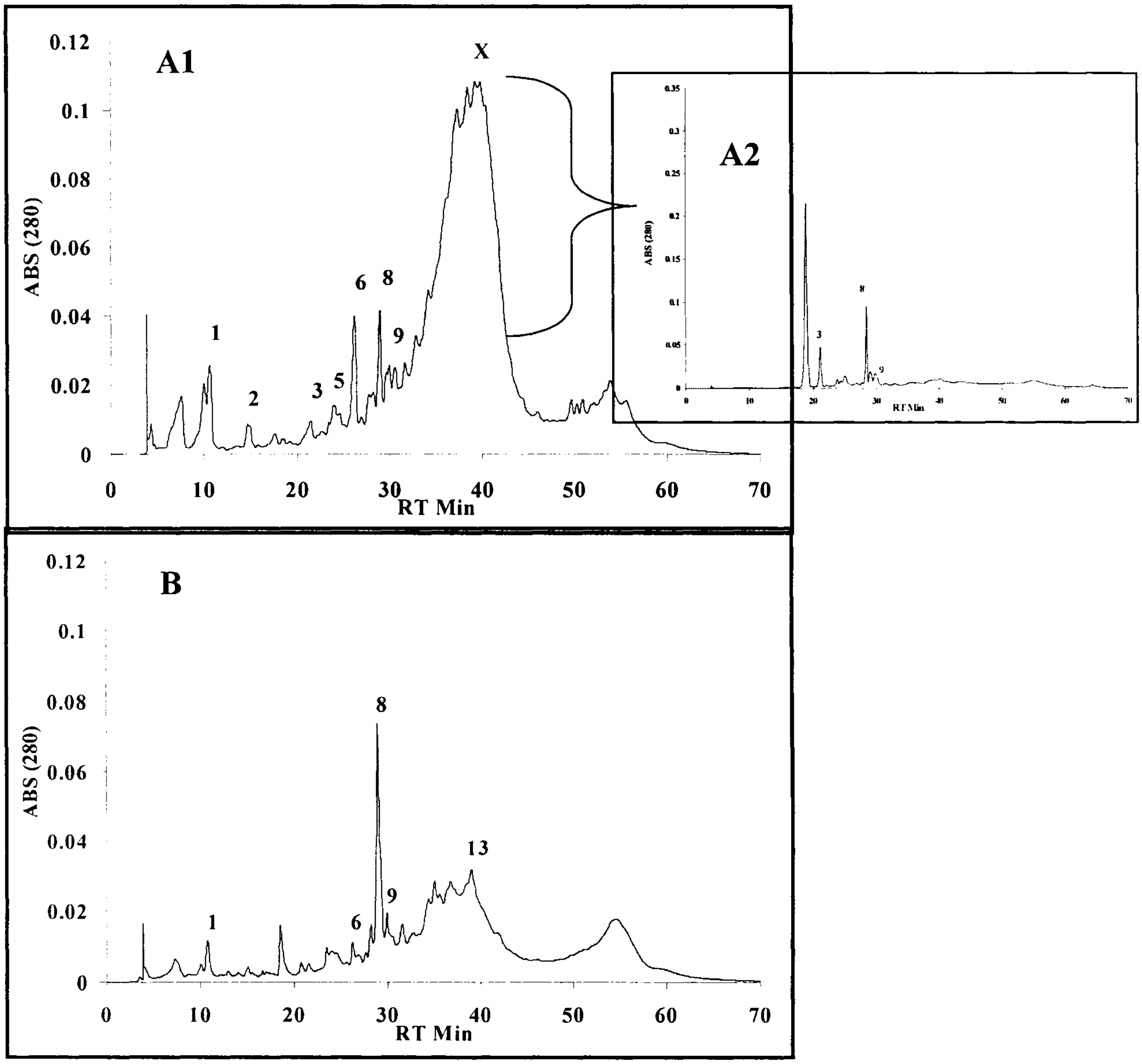


Figure 4.3: RP-HPLC of phenolic compounds for protein isolates from full-fat flaxseed; (A1) Free phenolic compounds; (A2) Fraction X after base hydrolysis; (B) Bound phenolic compounds extracted after base hydrolysis; Peaks: 1, Gallic acid; 2, Protocatechuic acid; 3, Hydroxybenzoic acid; 5, Caffeic acid; 6, Syringic acid; 8, Ferulic acid; 9, *p*-Coumaric acid; 13, Cinnamic acid.

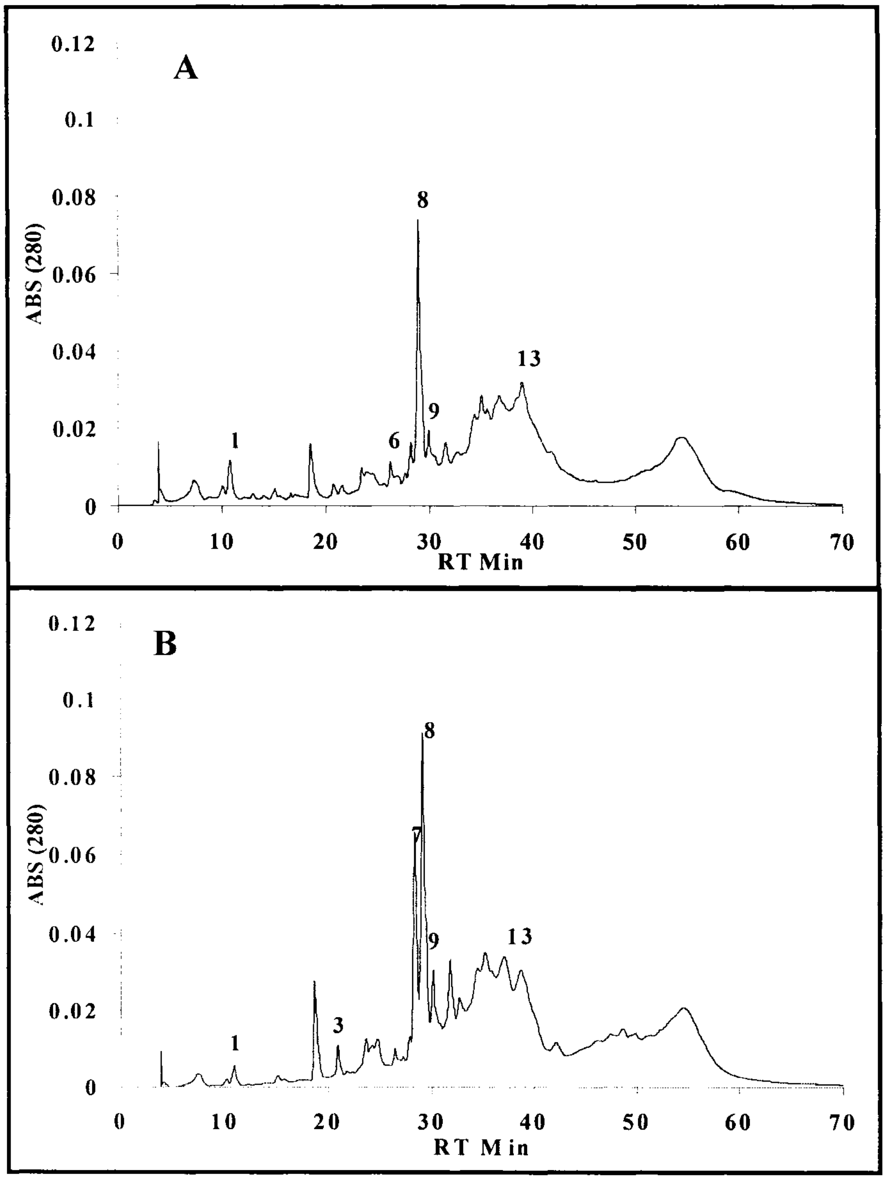


Figure 4.4: RP-HPLC of phenolic compounds for bound (base hydrolysis extraction) for protein isolates from; (A) Full- fat flaxseed; (B) Defatted flaxseed. Peaks: 1, Gallic acid; 3, Hydroxybenzoic acid; 7, Sinapic acid; 8, Ferulic acid; 9, *p*-Coumaric acid; 13, Cinnamic acid.

Table 4.2: Phenolic compounds identified by RP-HPLC for protein isolates (I) from full-fat (F) and defatted (D) soybean (s) and flaxseed (F).

Peak	Phenolic Compound	FsI		DsI		FfI		DfI	
#		Free	Bound	Free	Bound	Free	Bound	Free	Bound
1	Gallic Acid	X	-	X	-	X	X	X	X
2	Protocatechuic Acid	-	-	-	-	X	-	X	-
3	Hydroxybenzoic Acid	X	-	X	-	X	X	X	X
4	Vanillic Acid	-	-	-	-	-	-	-	-
5	Caffeic Acid	X	-	X	-	X	X	X	X
6	Syringic Acid	X	X	X	X	X	X	X	X
7	Sinapic Acid	X	-	X	-	-	-	-	X
8	Ferulic Acid	X	-	X	-	X	X	X	X
9	*p*-Coumaric Acid	X	X	X	X	X	X	X	X
10	Rutin	-	-	-	-	-	-	-	-
11	Hesperidin	X	X	X	X	-	-	-	-
12	Quercetin	X	-	X	-	-	-	-	-
13	Cinnamic Acid	-	-	-	-	-	X	-	X

X; Represent the presence of phenolic compounds; (-): Represent not identified.

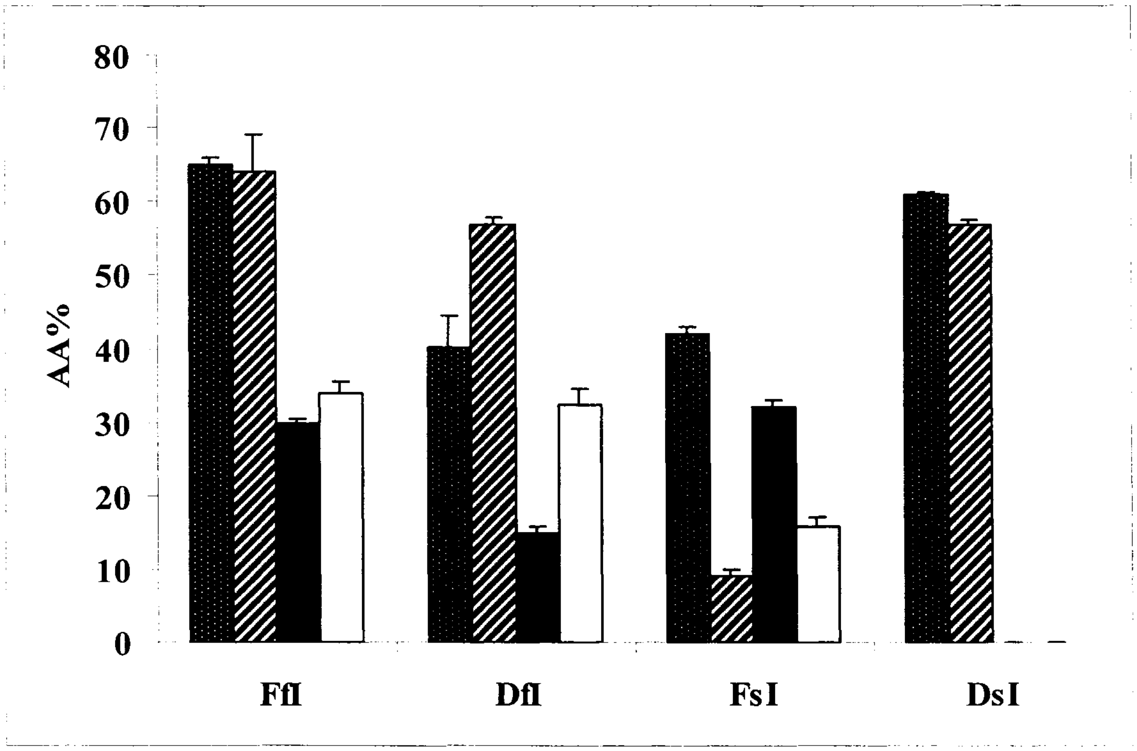


Figure 4.5: Antioxidant activity (AA%) of protein isolates (I) from full-fat (F) and defatted (D) soybean (s) and flaxseed (f). ▦ Free phenolic compounds (methanol/23°C extraction); ▨ Free phenolic compounds (methanol/60°C extraction); ■ Bound phenolic compounds (NaOH hydrolysis); □ Bound phenolic compounds (HCl hydrolysis).

Table 4.3: Antioxidant activity (AA%) of free and bound phenolic compounds of protein isolates (I) from full-fat (F) and defatted (D) soybean (s) and flaxseed (f).

Mode of Extraction	FfI	DfI	FsI	DsI
Free phenolic compounds extracted at room temperature	65.0±1.1	40.0±1.0	42.0±4.5	61.0±0.4
Free phenolic compounds extracted at 60°C temperature	64.0±5.2	57.0±1.0	9.0±0.9	57.0±0.5
Bound phenolic compounds extracted after base hydrolysis	30.0±0.6	15.0±0.9	32.0±1.0	0.0±0.0
Bound phenolic compounds extracted after acid hydrolysis	34.0±1.5	32.5±1.2	16.0±2.1	0.0±0.0

Results are means ± standard deviations of four determinations.

4.3.4 Effect of Removal of Phenolic Compounds on Protein Characteristics

4.3.4.1 Polyacrylamide Gel Electrophoresis

Figures 4.6 and 4.7 show the Native-PAGE and SDS-PAGE of soybean and flaxseed protein isolates before and after removal of phenolic compounds. For protein isolates from both full-fat and defatted soybean and flaxseed, Native-PAGE revealed that the circled major bands before removal of phenolic compounds were very minor bands after removal of free and bound phenolic compounds. This effect was more pronounced after removal of bound phenolic compounds. It is also possible that the effect may be due to the basic and acidic hydrolysis used for removal of bound phenolic compounds. SDS-PAGE (Figure 4.6 and 4.7) revealed that removal of free and bound phenolic compounds from protein isolates of full-fat and defatted flaxseed and soybean had little effect on the subunit structure of the proteins.

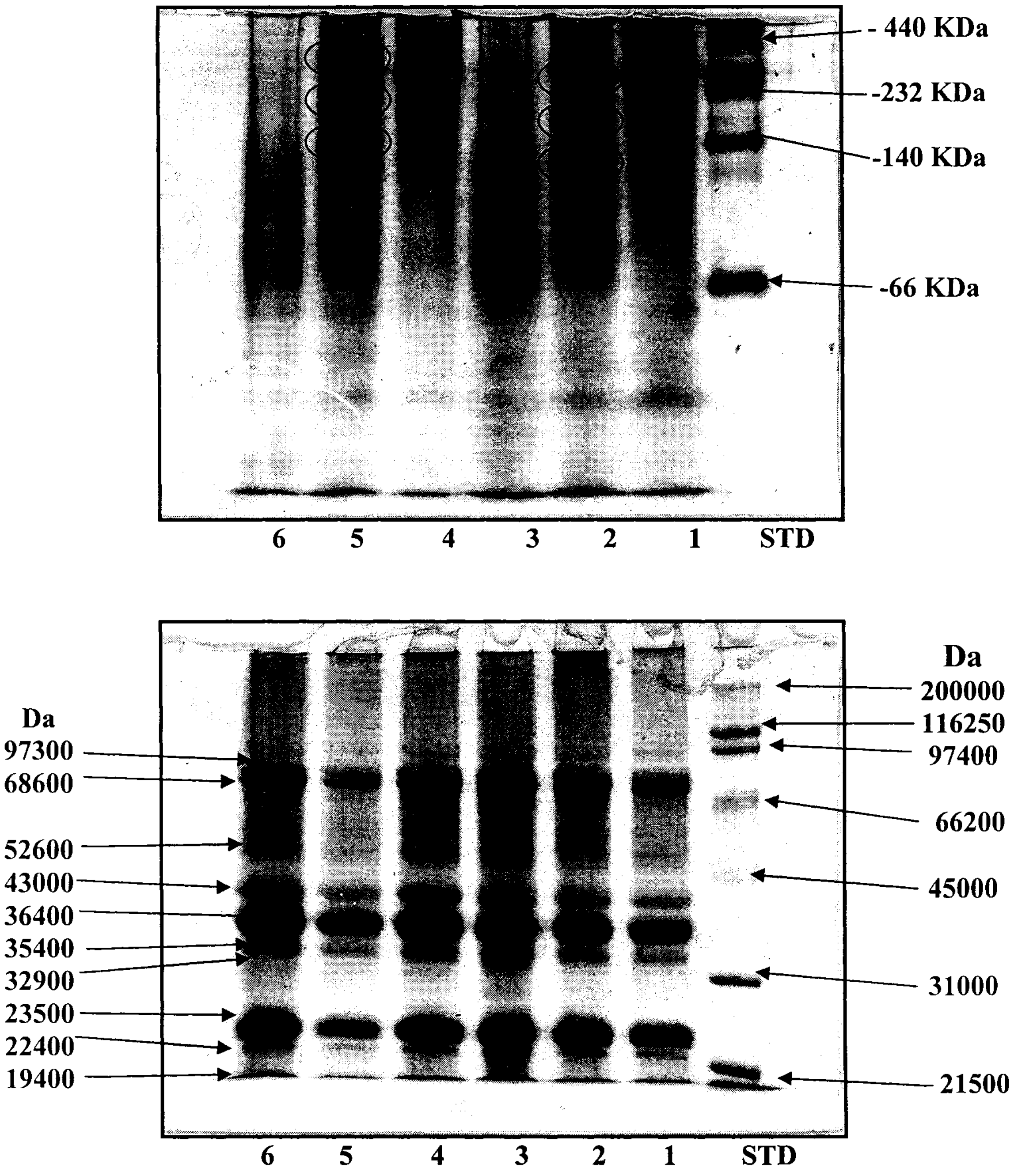


Figure 4.6: Native-PAGE and SDS-PAGE for protein isolate (I) from defatted (D) and full fat (F) soybean (s); (1) DsI; (2) DsI after removal of free phenolic compounds; (3) DsI after removal of free and bound phenolic compounds; (4) FsI; (5) FsI after removal of free phenolic compounds; (6) FsI after removal of free and bound phenolic compounds.

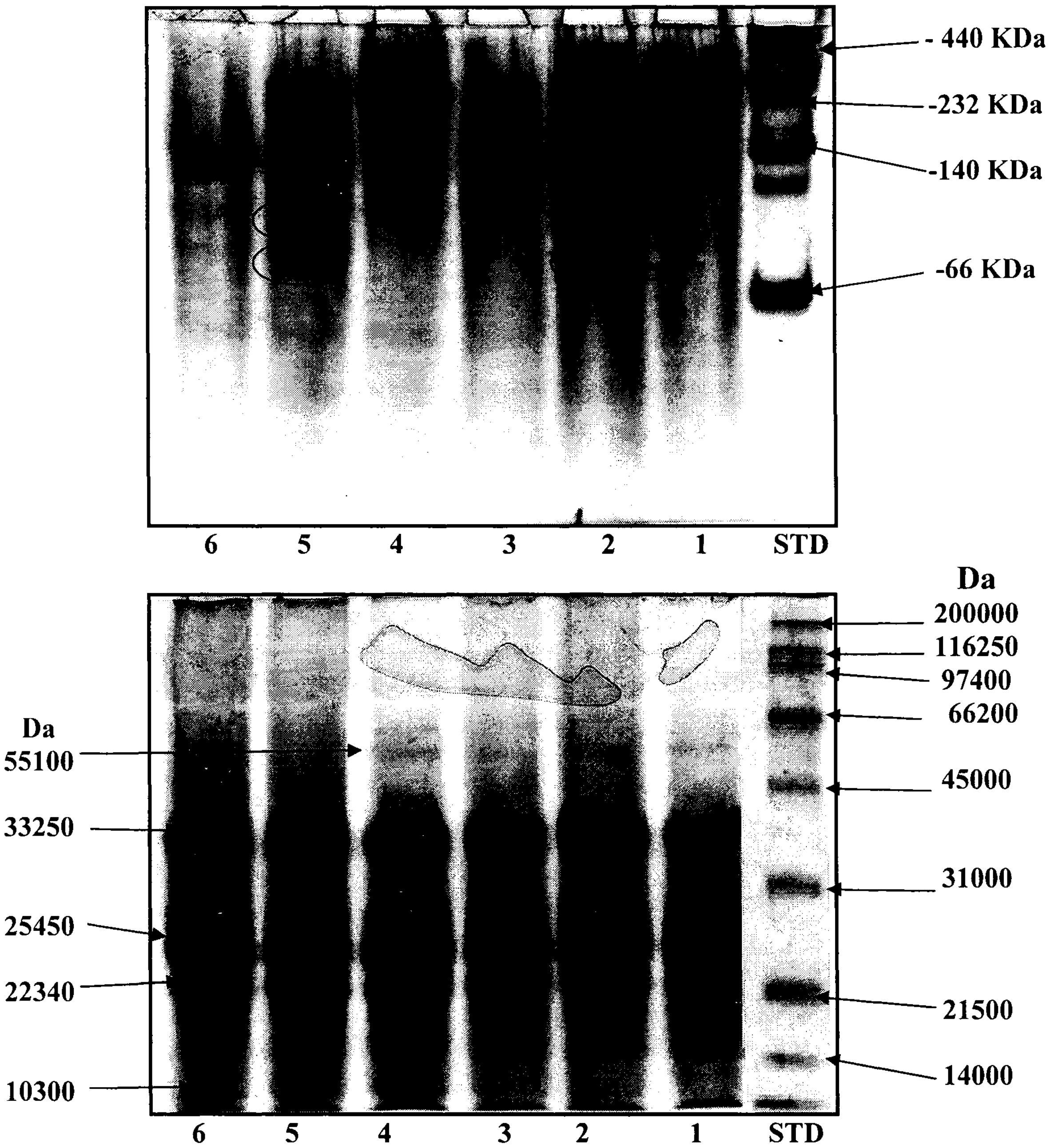


Figure 4.7: Native-PAGE and SDS-PAGE for protein isolate (I) from defatted (D) and full fat (F) for flaxseed (f); (1) DfI; (2) DfI after removal of free phenolic compounds; (3) DfI after removal of free and bound phenolic compounds; (4) FfI; (5) FfI after removal of free phenolic compounds; (6) FfI after removal of free and bound phenolic compounds.

4.3.4.2 RP-HPLC and Polyacrylamide Gel Electrophoresis

Figure 4.8 show the RP-HPLC chromatograms from protein isolates for full-fat soybean before and after removal of free and bound phenolic compounds; the chromatograms for protein isolates from full-fat soybean were similar to those from the defatted soybean. The chromatograms showed four major fractions; F1 (retention time 20.0-25.0 min), F2 (retention time 25.0-29.0 min), F3 (retention time 29.0-32.5 min), and F4 (retention time 32.5-40.0 min). Mujoo *et al.* (2003) reported that the soybean protein isolates were separated by RP-HPLC for 10 fractions. Proteins extracted from soybean protein isolates were separated by RP-HPLC into 17 fractions (Oomah *et al.*, 1994), 11 fractions (Wolf *et al.*, 1992) and 9 fractions (Castro-Rubio *et al.*, 2005).

SDS-PAGE (Figures 4.9 and Table 4.4) showed the presence of subunits with molecular weight 18.9 KDa in F1 from protein isolate of defatted soybean. For protein isolate from full-fat soybean, the F1 did not show the presence of the subunit 18.9 KDa. SDS-PAGE for F2 from protein isolates from full-fat and defatted soybean showed that the subunit with molecular weight 17.62 KDa; this subunits was not detected in the protein isolates after removal of phenolic compounds (Figure 4.10 and Table 4.4).

Figure 4.11 and Table 4.4 show SDS-PAGE for F3 of protein isolates from full-fat and defatted soybean before and after removal of free and bound phenolic compounds; 8 major subunits were obtained for protein isolate before and after removal of free phenolic compounds with molecular weight 41.9, 38.0, 35.7, 34.1 KDa, 29.1, 25.2, 24.3 and 22.4 KDa. For protein isolates from full-fat and defatted soybean after removal of free and bound phenolic compounds, 3 major subunits were obtained with molecular weight 41.9, 38.0 and 29.1 KDa. The subunits with molecular weight 35.7, 34.1, 25.2, 24.3 and 22.4

were absent after removal of free and bound phenolic compounds compared for protein isolates before removal of phenolic compounds and after removal of free phenolic compounds.

Figure 4.12 and Table 4.4 show SDS-PAGE of F4 from protein isolates from full-fat and defatted soybean before and after removal of free and bound phenolic compounds; 9 major subunits were obtained for F4 from protein isolate from defatted soybean with molecular weight 87.0, 52.9, 41.3, 37.6, 32.5, 29.2, 23.8, 18.2 and 14.8 KDa. For F4 from protein isolate from defatted soybean after removal of free and bound phenolic compounds, 8 major subunits were obtained with the absence of subunit with molecular weight 14.8.

F4 from protein isolates from full-fat soybean before removal of phenolic compounds and after removal of free and bound phenolic compounds (Figure 4.12 and Table 4.4) showed the absence of subunits with molecular weights 14.8 and 37.6 as compared to F4 from protein isolate from defatted soybean after removal of free and bound phenolic compounds.

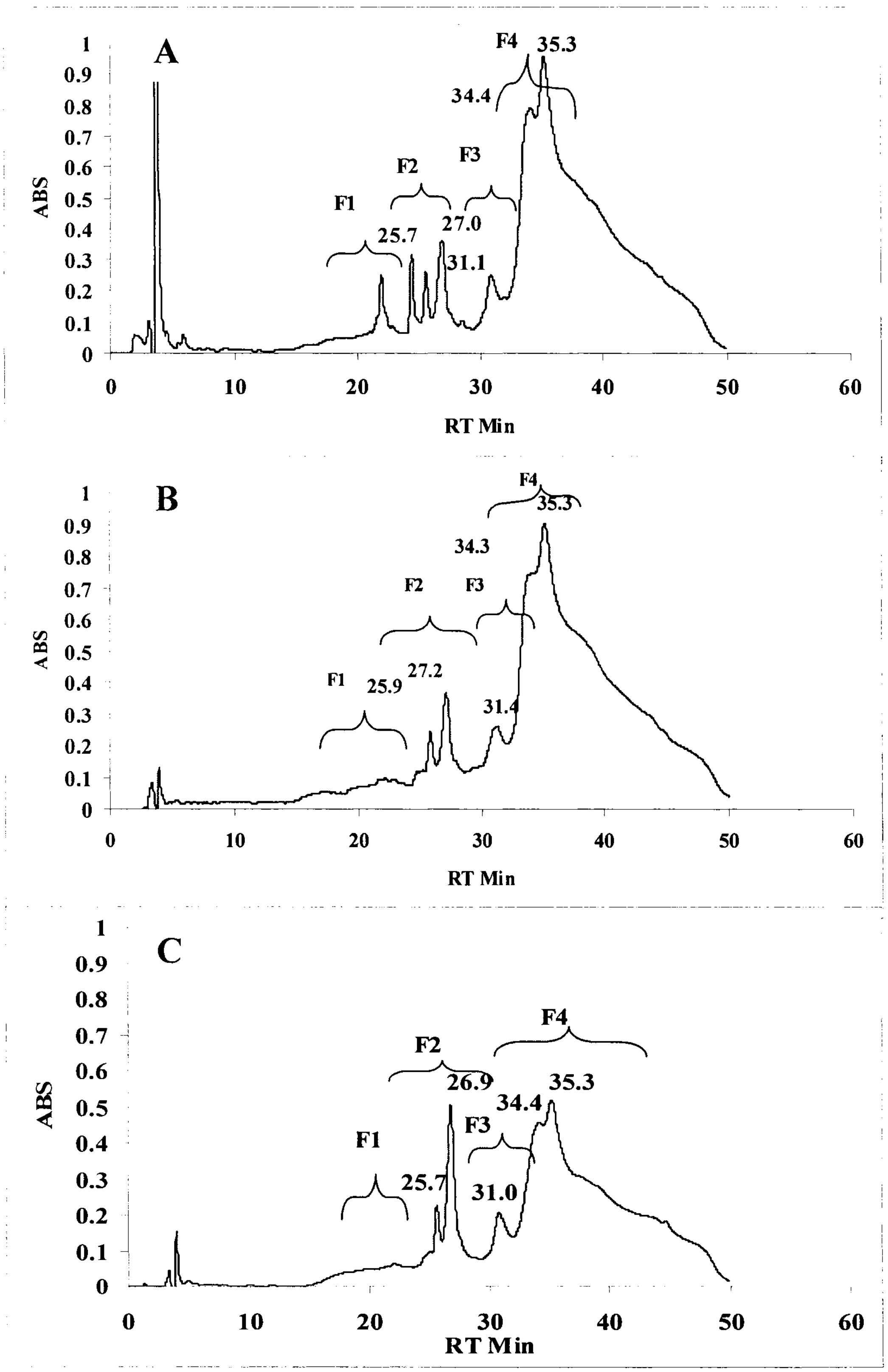


Figure 4.8: RP-HPLC separation for protein isolate (I) from full-fat (F) soybean (s); (A) FsI; (B) FsI after removal of free phenolic compounds; (C) FsI after removal of free and bound phenolic compounds.

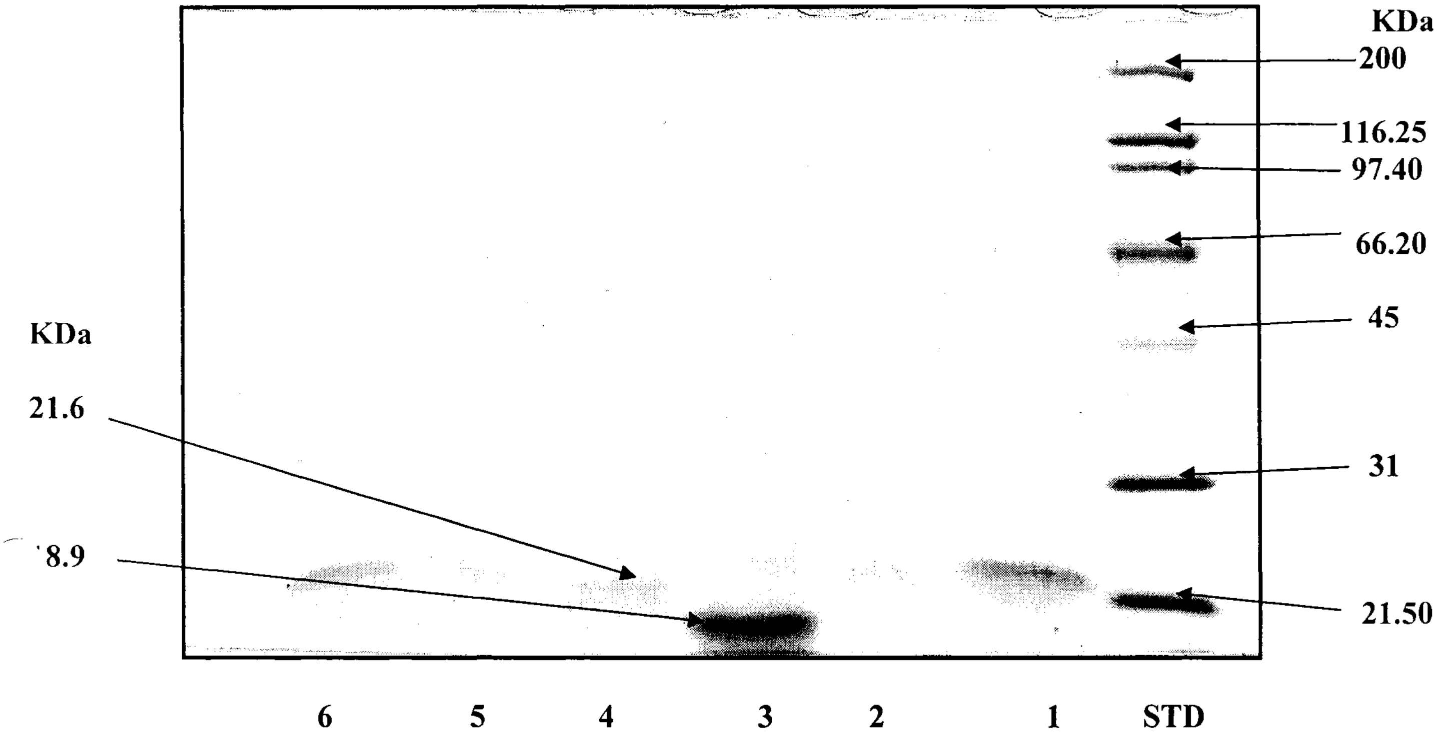


Figure 4.9: SDS-PAGE for F1 form protein isolate (I) of defatted (D) and full fat (F) soybean (s); (1) DsI; (2) DsI after removal of free phenolic compounds; (3) DsI after removal of free and bound phenolic compounds; (4) FsI; (5) FsI after removal of free phenolic compounds; (6) FsI after removal of free and bound phenolic compounds.

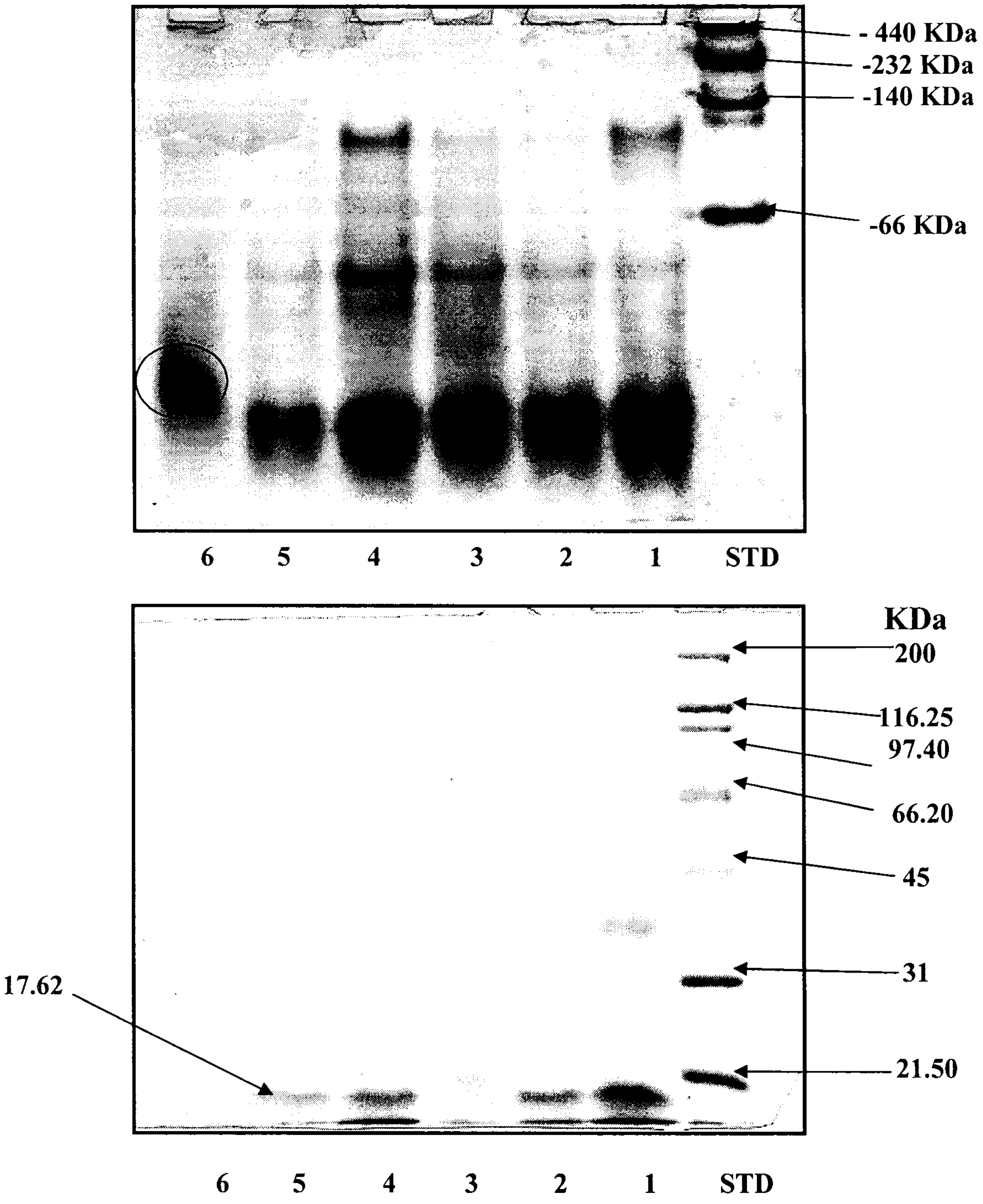


Figure 4.10: Native-PAGE and SDS-PAGE for F2 from protein isolate (I) of defatted (D) and full fat (F) soybean (s); (1) DsI; (2) DsI after removal of free phenolic compounds; (3) DsI after removal of free and bound phenolic compounds; (4) FsI; (5) FsI after removal of free phenolic compounds; (6) FsI after removal of free and bound phenolic compounds.

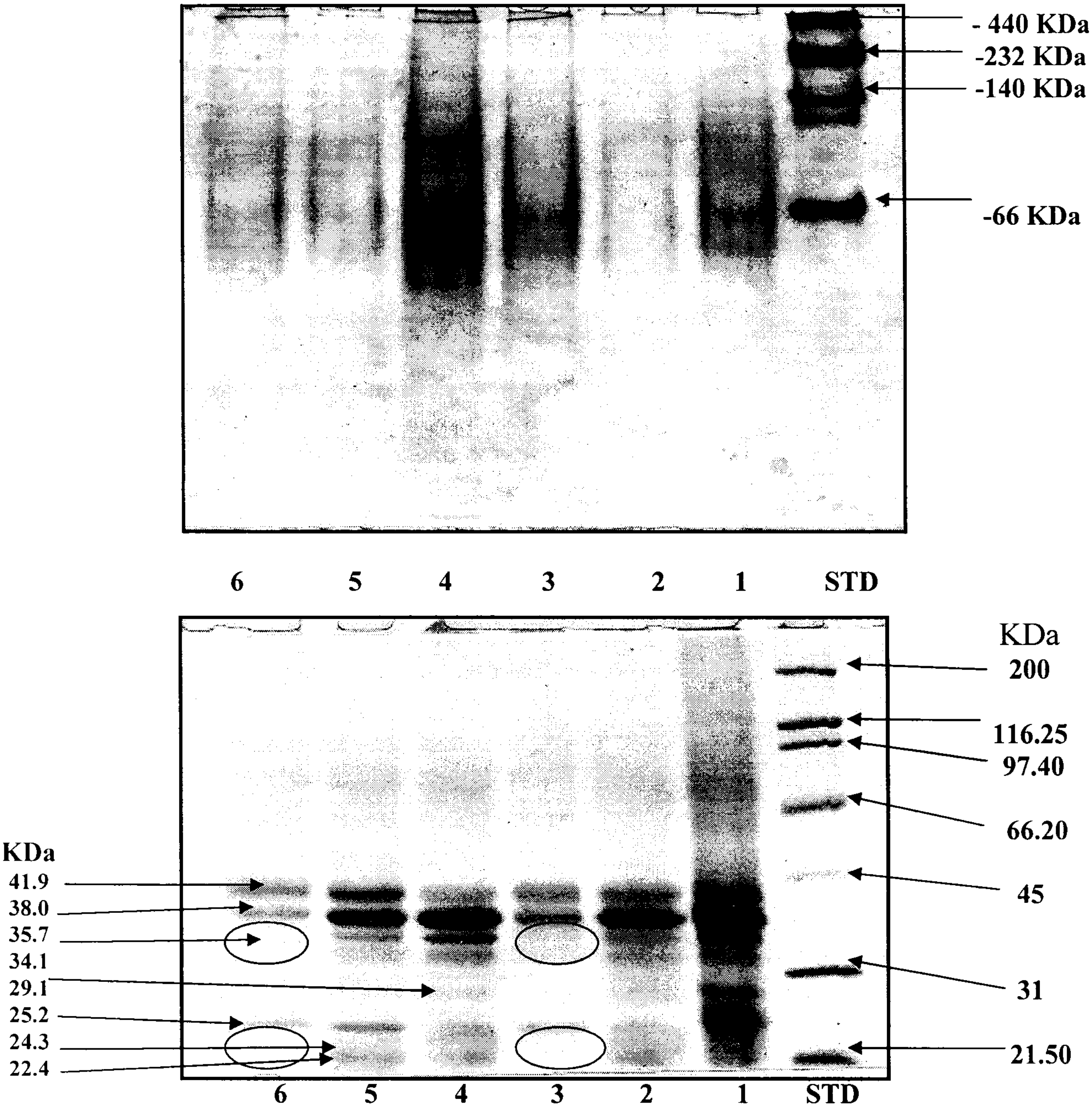


Figure 4.11: Native-PAGE and SDS-PAGE for F3 from protein isolate (I) of defatted (D) and full fat (F) soybean (s); (1) DsI; (2) DsI after removal of free phenolic compounds; (3) DsI after removal of free and bound phenolic compounds; (4) FsI; (5) FsI after removal of free phenolic compounds; (6) FsI after removal of free and bound phenolic compounds.

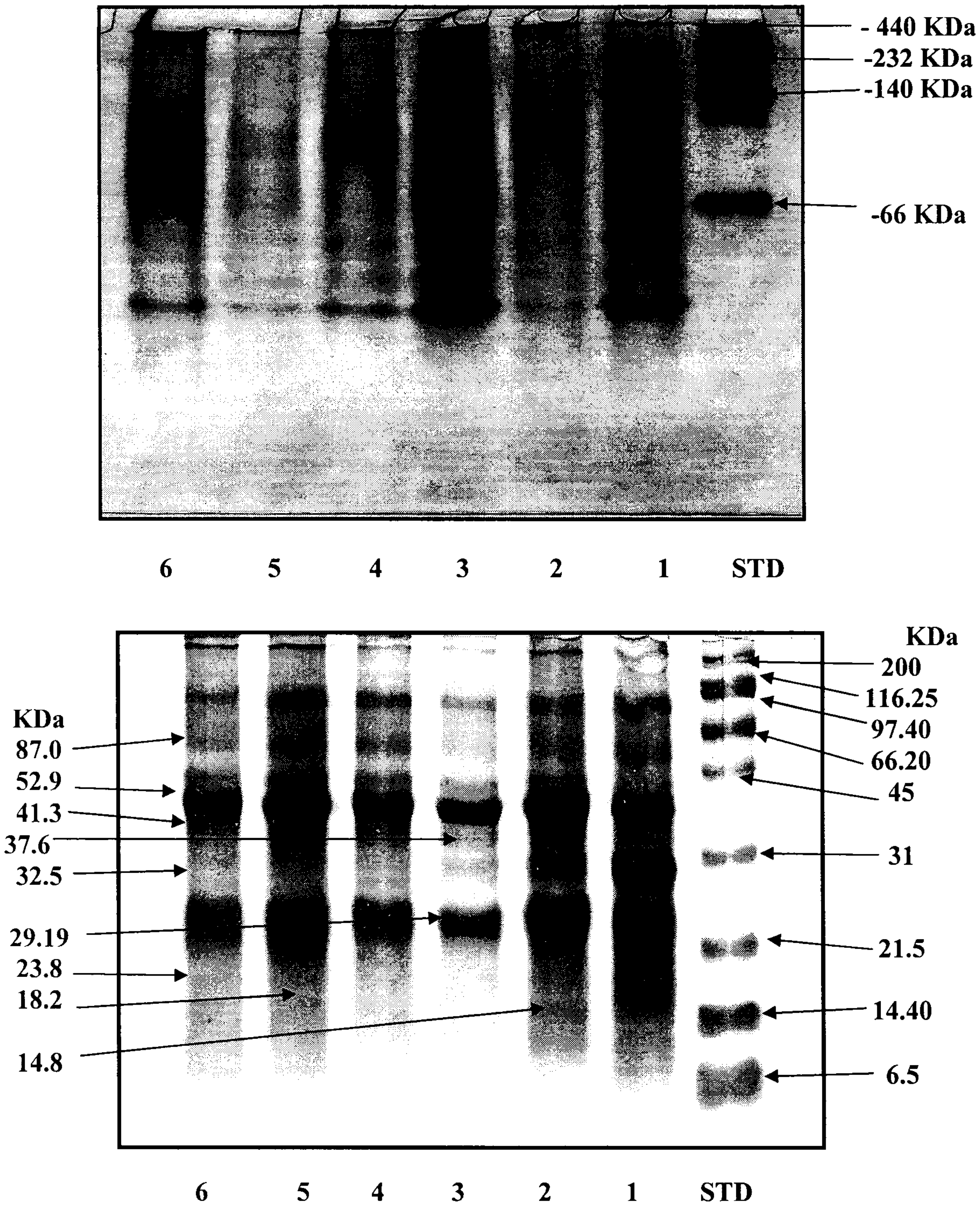


Figure 4.12: Native-PAGE and SDS-PAGE for F4 from protein isolate (I) of defatted (D) and full fat (F) soybean (s); (1) DsI; (2) DsI after removal of free phenolic compounds; (3) DsI after removal of free and bound phenolic compounds; (4) FsI; (5) FsI after removal of free phenolic compounds; (6) FsI after removal of free and bound phenolic compounds.

Table 4.4: Molecular weights (KDa) from SDS of F1, F2, F3 and F4 before and after removal of free and bound phenolic compounds separated by RP-HPLC from protein isolate (I) for defatted (D) and full fat (F) soybean (s).

Treatment	Subunits Molecular Weight			
	F1	F2	F3	F4
DsI before removal of phenolic compounds	21.6	17.62	41.9, 38.0, 35.7, 34.1 29.1, 25.2, 24.3, 22.4	87.0, 52.9, 41.3, 37.6, 32.5 29.19, 23.8, 18.2, 14.8
DsI after removal of free phenolic compounds	21.6	17.62	41.9, 38.0, 35.7, 34.1 29.1, 25.2, 24.3, 22.4	87.0, 52.9, 37.6, 32.5 29.19, 23.8, 18.2, 14.8
DsI after removal of both free and bound phenolic compounds	18.9	ND	41.9, 38.0 29.1	87.0, 52.9, 41.3, 37.6, 32.5, 29.19, 23.8, 18.2
FsI before removal of phenolic compounds	21.6	17.62	41.9, 38.0, 35.7, 34.1 29.1, 25.2, 24.3, 22.4	87.0, 52.9, 41.3, 37.6, 32.5, 23.8, 18.2
FsI after removal of free phenolic compounds	21.6	17.62	41.9, 38.0, 35.7, 34.1 29.1, 25.2, 24.3, 22.4	87.0, 52.9, 41.3, 37.6, 32.5, 23.8; 18.2
FsI after removal of both free and bound phenolic compounds	21.6	ND	41.9, 38.0 29.1	87.0, 52.9, 41.3, 37.6, 32.5, 23.8, 18.2

ND: Not Detected.

Figures 4.13 show the RP-HPLC chromatograms for protein isolates from full-fat flaxseed. Chromatograms for protein isolates from full-fat flaxseed were similar to those for protein isolates from defatted flaxseed. The chromatograms show two major fractions: F1 (retention time 20.0-30.0 min) and F2 (retention time 30.0-40.0 min). El-ramahi (2003) reported the presence of 3 major fractions from RP-HPLC for protein extracts from flaxseed.

Figures 4.14 and 4.15 and Tables 4.5 show SDS-PAGE and Native-PAGE of F1 and F2 for protein isolates from full-fat and defatted flaxseed before and after removal of phenolic compounds, the identified subunits were: 2 subunits for F1 with molecular weight 13.3 and 9.5 KDa for protein isolate from defatted and 13.8 and 12.3 KDa for protein isolate from full-fat (Figure 4.14 and Table 4.5) and 6 subunits for F2 with molecular weight 37.5, 29.2, 26.2, 24.5, 16.4 and 13.5 KDa for protein isolate from full-fat and 36.4, 28.6, 23.6, 22.0, 13.6 and 10.5 KDa for protein isolate from defatted (Figure 4.15 and Table 4.5).

The removal of free and bound phenolic compounds showed little effect on the electrophoretic behavior (Native-PAGE and SDS-PAGE) for flaxseed proteins and their protein subunits (Figures 4.14 and 4.15 and Table 4.5). Oomah and Mazza (1998) reported the presence of 4 subunits in flaxseed with molecular weight 14, 24, 25 and 34 KDa. While Marcone *et al.* (1998) reported five major subunits with molecular weight 14.4, 24.6, 30, 35.2 and 50.9 KDa. Marcone *et al.* (1998) found that the protein fractions contain subunits with molecular weight 20-27 KDa and 30-39 KDa. Madhusudhan and Singh (1983) reported 5 major subunits with molecular weight 11.0, 18.0, 29.0, 42.0 and 61.0 KDa. El-ramahi (2003) reported that protein isolate was fractionated by RP-HPC into 3 subunits with molecular weight 10.3, 25.6 and 36 KDa.

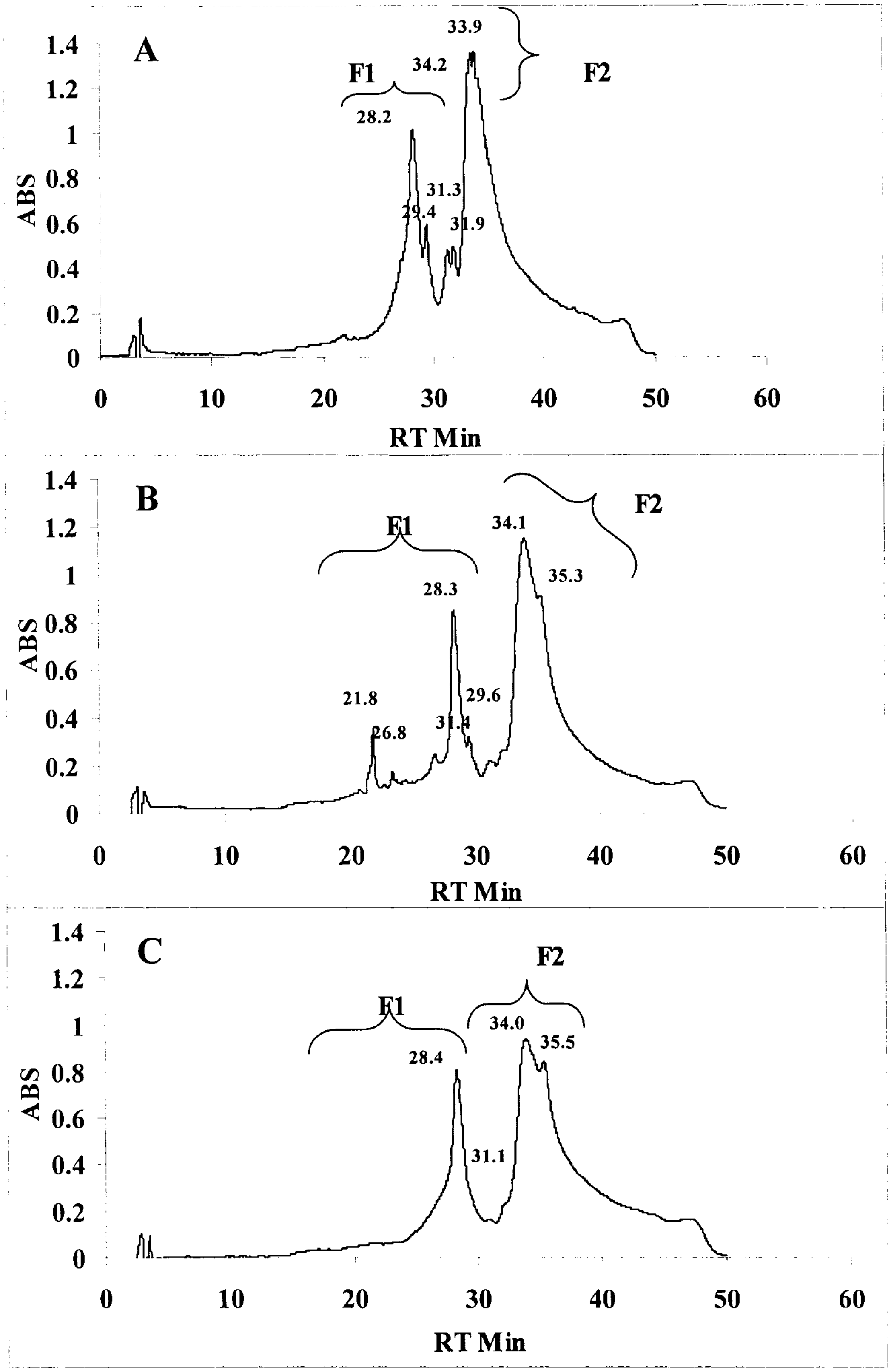


Figure 4.13: RP-HPLC separation for protein isolate (I) from full-fat (f) flaxseed (f); (A) FfI; (B) FfI after removal of free phenolic compounds; (C) FfI after removal of free and bound phenolic compounds.

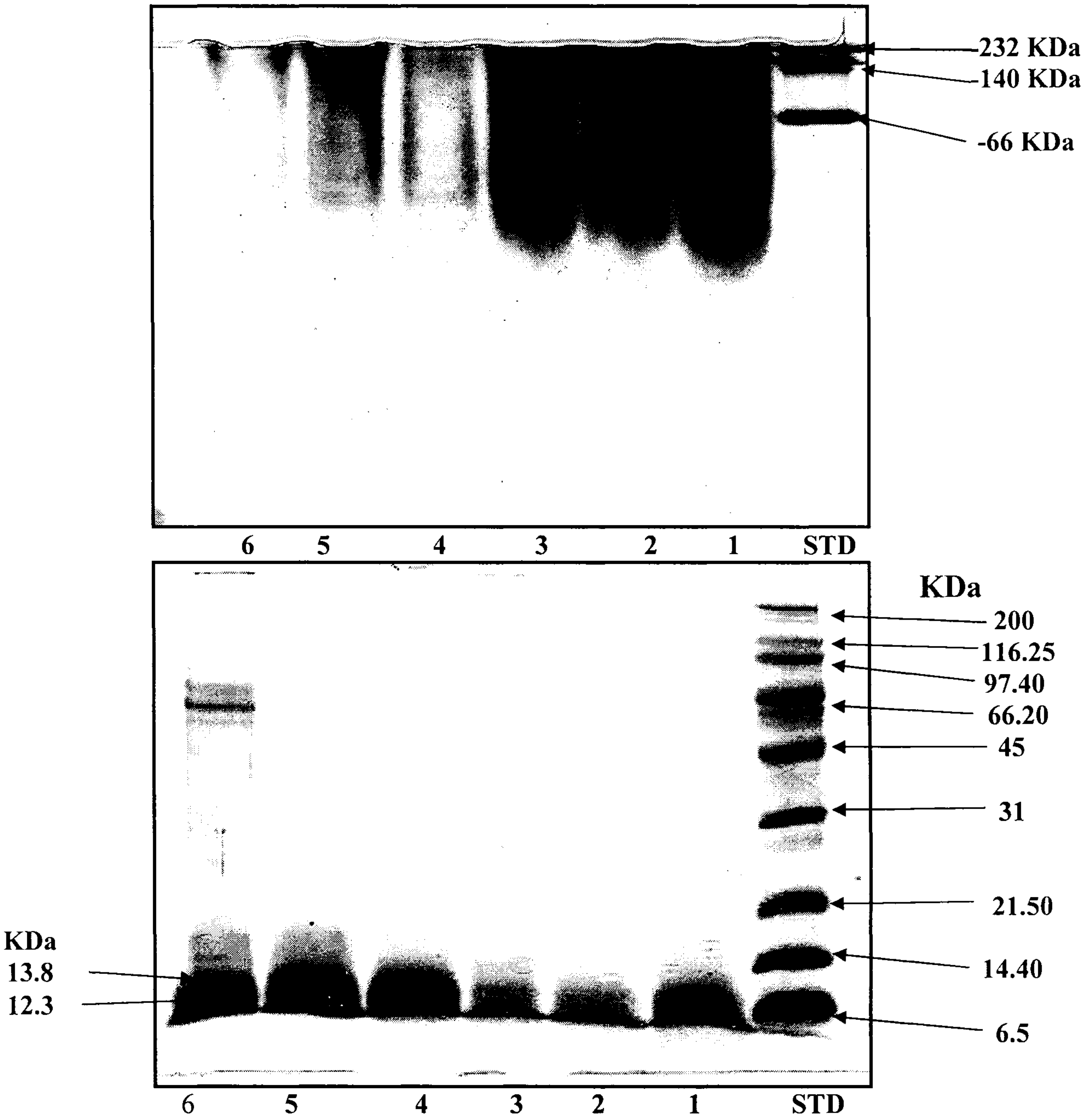


Figure 4.14: SDS and Native PAGE for F1 from protein isolate (I) of defatted (D) and full fat (F) flaxseed (f); (1) DfI; (2) DfI after removal of free phenolic compounds; (3) DfI after removal of free and bound phenolic compounds; (4) FfI; (5) FfI after removal of free phenolic compounds; (6) FfI after removal of free and bound phenolic compounds.

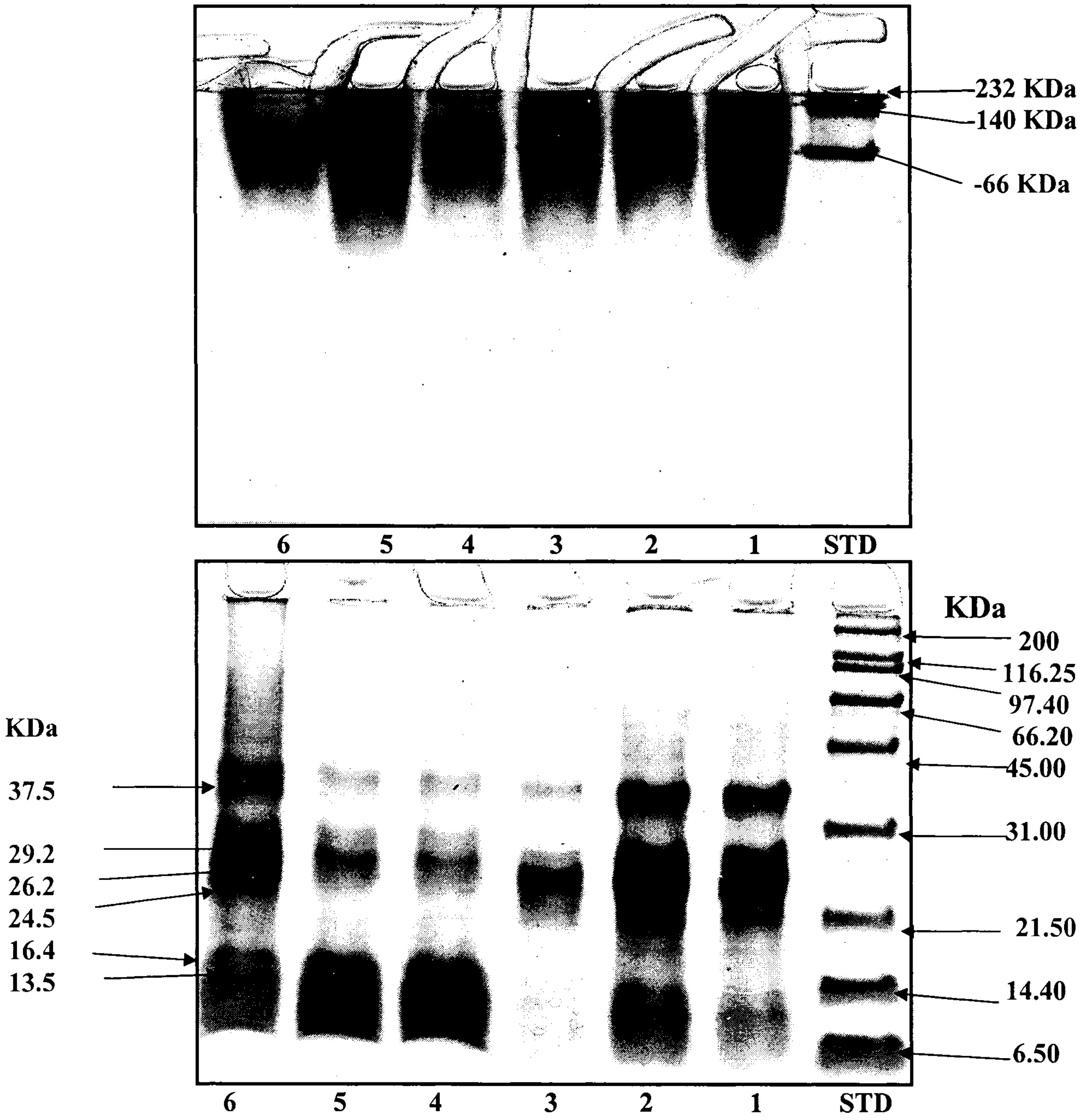


Figure 4.15: SDS and Native PAGE for F2 from protein isolate (I) of defatted (D) and full fat (F) flaxseed (f); (1) DfI; (2) DfI after removal of free phenolic compounds; (3) DfI after removal of free and bound phenolic compounds; (4) FfI; (5) FfI after removal of free phenolic compounds; (6) FfI after removal of free and bound phenolic compounds.

Table 4.5: Molecular weights (KDa) from SDS of protein isolate (I) from defatted (D) and full fat (F) flaxseed (f) for F1 and F2 before and after removal of free and bound phenolic compounds separated by RP-HPLC.

Treatment	Subunits Molecular Weight	
	F1	F2
DfI before removal of phenolic compounds	13.3, 9.5	36.4, 28.6, 23.6, 22.0, 13.6, 10.5
DfI after removal of free phenolic compounds	13.3, 9.5	36.4, 28.6, 23.6, 22.0, 13.6, 10.5
DfI after removal of free and bound phenolic compounds	13.3, 9.5	36.4, 28.6, 23.6, 22.0, 13.6, 10.5
FfI before removal of phenolic compounds	13.8, 12.3	37.5, 29.2, 26.2, 24.5, 16.4, 13.5
FfI after removal of free phenolic compounds	13.8, 12.3	37.5, 29.2, 26.2, 24.5, 16.4, 13.5
FfI after removal of free and bound phenolic compounds	13.8, 12.3	37.5, 29.2, 26.2, 24.5, 16.4, 13.5

4.3.4.3 Electrospray Ionization Mass Spectrometry

Figures 4.16 and 4.17 and Table 4.6 show mass spectra of fraction F4 for protein isolates from full-fat and defatted soybean before and after removal of free and bound of phenolic compounds; the identification of subunits are based on SDS-PAGE, for soybean protein isolates before removal of phenolic compounds. The protein found in this fraction gave 11 subunits with molecular weight 13 025, 16 700, 19 956, 21 324, 21 360, 23 294, 23 530, 35 510, 36 647, 36 734 and 37 742 Da; for fraction F4 from protein isolate from defatted soybean and 13 639, 15 880, 20 205, 21 067, 22 278, 22 950, 24 540, 37 161, 37 535, 39 178 and 39 799 Da for fraction F4 from protein isolate from full-fat soybean. The effect of removal of free phenolic compounds results a decrease in molecular weight for protein isolates from defatted soybean compared to control sample. The MS results for fraction F4 from protein isolates from full-fat soybean before removal of phenolic compounds were similar to protein isolates after removal of free phenolic compounds except for subunits 13 639 and 20 205. The effects of removal of free and bound phenolic compounds show molecular weights of 11 878, 16 425, 17 892, 18 692, 18 728, 21 101, 21 212, 33 053, 34 326, 35 507 and 35 623 Da for fraction F4 from protein isolate from defatted soybean, and 11 370, 15 059, 18 577, 19 495, 21 068, 22 208, 22 952, 35 510, 37 077, 38 033, and 39 621 Da for fraction F4 from protein isolate from full-fat soybean (Figure 4.16 and 4.17 and Table 4.6).

Figures 4.18 and 4.19 and Table 4.7 show the MS results for fraction F2 from protein isolates from full-fat and defatted flaxseed. MS of F2 for protein isolate before removal of phenolic compounds from full-fat flaxseed was similar to that of F2 from protein isolate after removal of free phenolic compounds (Figures 4.18 and 4.19 and Table 4.7). The removal of free and bound phenolic compounds for full-fat and defatted protein isolates showed a decrease in molecular weight as compared to protein isolates before removal of phenolic compounds and after removal of free phenolic compounds. Protein isolate before removal of free and bound phenolic compounds showed molecular weights ND, 23 179, 26 745, 33 339, 38 437, and 40 807 Da for fraction F2 from protein isolate from defatted flaxseed, and 15 605, 23 986, 26 613, 30 544, 37 502, and 39 074 Da for fraction F2 from protein isolate from full-fat flaxseed. Removal of free and bound phenolic compounds for fraction F4 from protein isolate from flaxseed gave molecular weight of 15 231, 22 540, 25 535, 30 500, 34 607 and 38 246 Da for defatted, and 15 581, 23 567, 25 312, 30 000, 36 309 and 38 771 Da for full-fat (Figures 4.18 and 4.19 and Table 4.7).

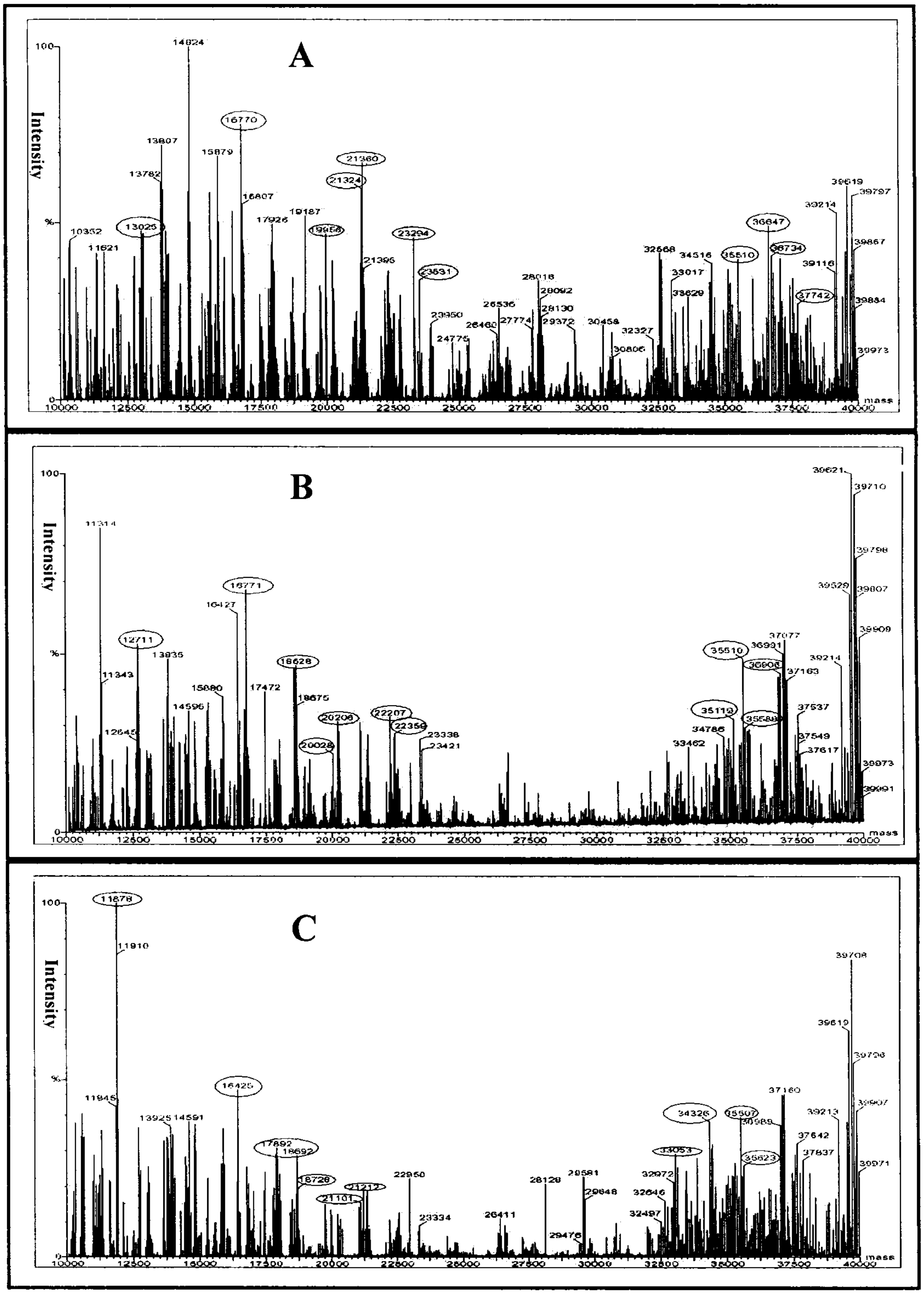


Figure 4.16: ESI-MS Spectra for fraction F4 from protein isolate (I) from defatted (D) soybean (s); (A) DsI; (B) DsI after removal of free phenolic compounds; (C) DsI after removal of free and bound phenolic compounds.

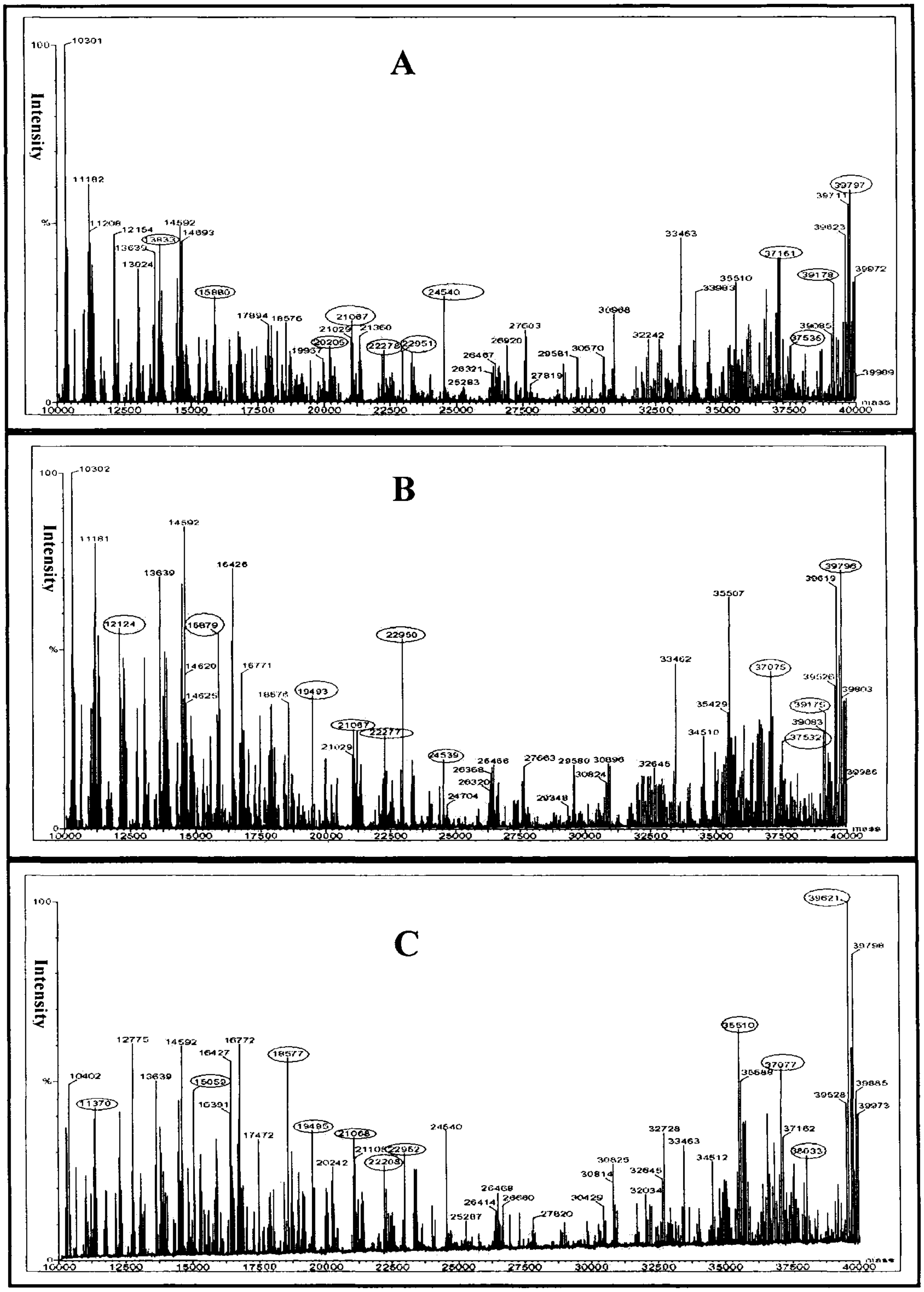


Figure 4.17: ESI-MS Spectra for fraction F4 from protein isolate (I) from full-fat (F) soybean (s); (A) FsI; (B) FsI after removal of free phenolic compounds; (C) FsI after removal of free and bound phenolic compounds.

Table 4.6: Molecular weight MW (Da) of protein subunits before and after removal of free and bound phenolic compounds for fraction F4 from protein isolate (I) from defatted (D) and full-fat (f) soybean (s) from ESI-MS.

	Observed MW before Removal of Phenolic Compounds			Observed MW after Removal of Free Phenolic Compounds		Observed MW after Removal of Free and Bound phenolic Compounds	
	DsI	FsI	Literature Values	DsI	FsI	DsI	FsI
A_5	13 025	13 639	10 000-11750[a]	12 711	12 124	11 878	11 370
KTI	16 770	15 880	18 000[b]	16 771	15 879	16 425	15 059
B_{1a}	19 956	20 205	22 000-24 000[c]	18 628	19 493	17 892	18 577
B_{1b}	21 324	21 067	22 000-24 000[c]	20 028	21 067	18 692	19 495
B_2	21 360	22 278	22 000-24 000[c]	20 206	22 277	18 728	21 068
B_3	23 294	22 950	22 000-24 000[c]	22 207	22 950	21 101	22 208
B_4	23 530	24 540	22 000-24 000[c]	22 359	24 539	21 212	22 952
A_1	35 510	37 161	35 000-38 000[d]	35 119	37 075	33 053	35 510
A_2	36 647	37 535	35 000-38 000[d]	35 510	37 532	34 326	37 077
A_3	36 734	39 178	35 000-38 000[d]	35 558	39 175	35 507	38 033
A_4	37 742	39 799	35 000-38 000[d]	36 906	39 796	35 623	39 621

a: Sathe *et al.* (1987); Fontes *et al.* (1984), b: Sathe *et al.* (1989), c: Sathe *et al.* (1987); Moreira *et al.* (1979); Fonte *et al.* (1984), d: Sathe *et al.* (1987); Fonte *et al.* (1984); Moreia *et al.* (1979); Utsumi *et al.* (1985).

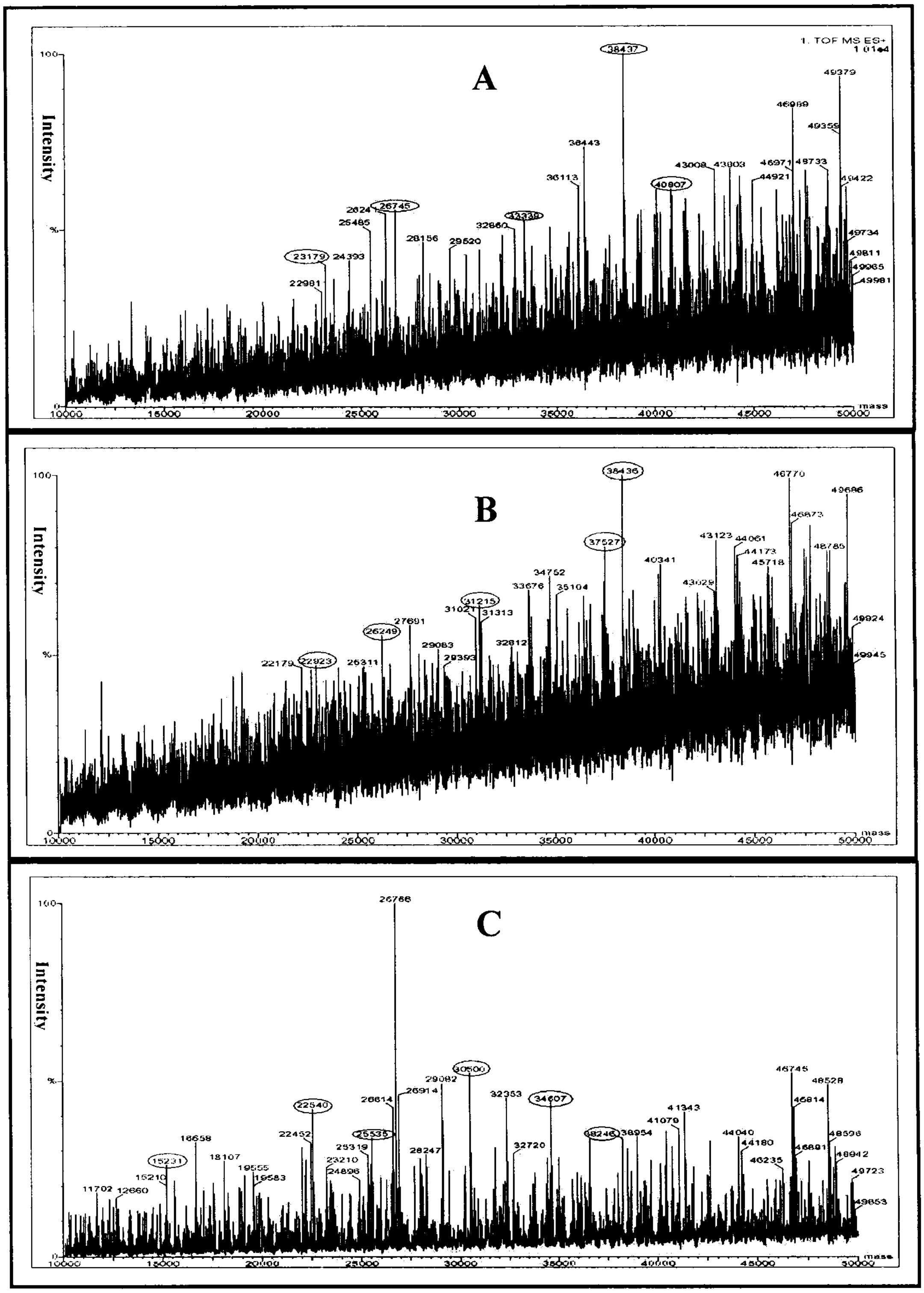


Figure 4.18: ESI-MS Spectra for fraction F2 from protein isolate (I) from defatted (D) flaxseed (f); (A) DfI; (B) DfI after removal of free phenolic compounds; (C) DfI after removal of free and bound phenolic compounds.

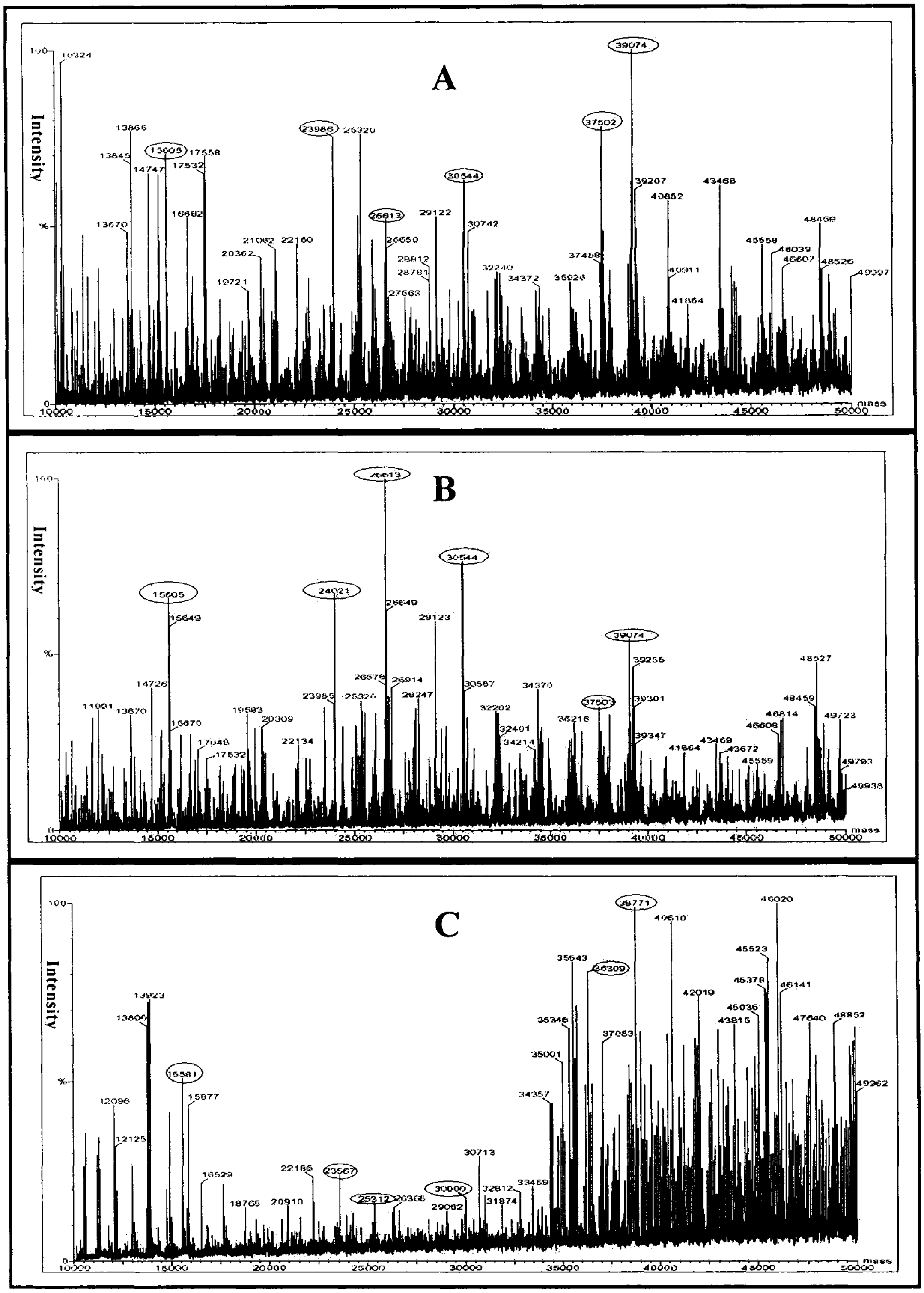


Figure 4.19: ESI-MS Spectra for fraction F2 from protein isolate (I) from full-fat (f) flaxseed (f); (A) FfI; (B) FfI after removal of free phenolic compounds; (C) FfI after removal of free and bound phenolic compounds.

Table 4.7: Molecular weight MW (Da) of protein subunits before and after removal of free and bound phenolic compounds for fraction F2 from protein isolate (I) from defatted (D) and full-fat (f) flaxseed (f) using ESI-MS.

Observed MW before Removal of Phenolic Compounds			Observed MW after Removal of Free Phenolic Compounds		Observed MW after Removal of Free and Bound phenolic Compounds	
DfI	**FfI**	**Literature Values**	**DfI**	**FfI**	**DfI**	**FfI**
ND	15 605	14 000-15 000[a]	NO	15 605	15 231	15 581
23 179	23 986	20 000-29 000[b]	22 923	23 985	22 540	23 567
26 745	26 613	20 000-29 000[b]	26 249	26 613	25 535	25 312
33 339	30 544	30 000-39 000[c]	31 215	30 544	30 500	30 000
38 437	37 502	30 000-39 000[c]	37 527	37 503	34 607	36 309
40 807	39 074	40 000-42 000[d]	38 436	39 074	38 246	38 771

Not Detected

a: Marcone *et al.* (1998); Oomah and Mazza (1998), b: El-ramahi (2003); Madhusudhan and Singh (1983); Chung *et al.* (2005); Marcone *et al.* (1998); Oomah and Mazza (1998), c: El-ramahi (2003); Chung *et al.* (2005); Marcone *et al.* (1998); Oomah and Mazza (1998), d: Madhusudhan and Singh (1983); Chung *et al.* (2005).

CHAPTER 5

EFFECT OF REMOVAL OF PHENOLIC COMPOUNDS ON PROTEOLYSIS OF SOYBEAN AND FLAXSEED PROTEINS

5.1 Justification

This Chapter addresses the third objective of this study which was to investigate the effect of removal of free and bound phenolic compounds on biological properties of protein isolates from soybean and flaxseed. Protein isolates from soybean and flaxseed were prepared using NaOH extraction and isoelectric precipitation followed by removal of free and bound phenolic compounds; the protein isolates were subjected to in vitro enzymatic hydrolysis using sequential gastrointestinal enzymes (pepsin and pancreatic enzymes trypsin and chymotrypsin). The molecular characterizations of peptides obtained from hydrolyzed protein isolates were studies using RP-HPLC, SDS-PAGE and Native-PAGE.

5.2 Materials and Methods

5.2.1 Materials

Protein isolates from defatted and full-fat soybean and flaxseed were used; removal of free and bound phenolic compounds from the protein isolates is described in Sections 4.2.2, 4.2.3 and 4.2.4.

5.2.2 Enzymatic Hydrolysis of Protein Isolates

Protein isolates (4% in distilled water, pH 2.0, 0.1 N HCl) were hydrolyzed according to the method described by Megias *et al.* (2004) and Vermeirssen *et al.* (2003) with some modifications, using gastrointestinal enzymes (pepsin and pancreatic enzymes trypsin and chymotrypsin; enzyme/substrate ratio 1:30 W/W). The mixture of pepsin enzyme and substrate was acidified to pH 2.0 using 0.1 N HCl, heated to 37°C and incubated and holding for 3 h followed by digestion with trypsin and chymotrypsin (1:1 W/W) for another 3 h at pH 7.5 and 37°C. The mixture was centrifuged (10,000 X g, 10 min), the supernatant were filtered with cheese cloth, cooled immediately, then lyophilized.

5.2.3 Polyacrylamide Gel Electrophoresis (PAGE)

Hydrolyzed protein isolates from Section 5.2.2 were subjected to Native-PAGE conditions according to the method described in Section 4.2.8.1 and SDS-PAGE according to the method described in Section 4.2.8.2.

5.2.4 RP-HPLC Analysis of Hydrolyzed Peptides

Hydrolyzed protein isolates from section 5.2.2 were mixed with 0.1 TFA% at concentration 10 mg/ml and centrifuged (5,000 X g, 10 min). The supernatants were filtered with (0.45 μm Millipore^{TM} membrane filters), and stored at −18°C. The supernatants were subjected to RP-HPLC analysis according to the method described by (Antila *et al.*, 1991). Analysis was carried out with a Beckman (CA, USA) liquid chromatography equipped with a Programmable Solvent Module (model 126) for high-pressure solvent delivery, a Beckman manual injector with a 100 μl loop and a Programmable Detector Module (Model 166). Spectral and chromatographic data were analyzed by the Gold System (version V810), translated into PRN format for Microsoft Excels manipulation and stored on disc. For chromatographic separation, 100 μl of the sample was injected into a protein and peptide C18 reversed phase column (micron, 250 X 4.6 mm, J.T Baker Inc., US) operated at room temperature. The sample was eluted at a flow rate of 0.8 ml/ min with following two buffer gradient system: solvent A, 0.1% TFA in water (V/V); solvent B, 0.11%TFA (60% acetonitrile/ 40% acidified water), starting increasing linearly from 0% to 90% over 50 min. The UV detector was calibrated and the elute was monitored at 215 nm.

5.3 Results and Discussion

5.3.1 Native-PAGE and SDS-PAGE of Hydrolyzed Protein Isolates

Figures 5.1 and 5.2 show Native-PAGE of hydrolyzed protein isolates from full-fat and defatted soybean before and after removal of free and bound phenolic compounds. The circled major bands in Figures 5.1 and 5.2 bands were absent for hydrolyzed protein isolates before and after removal of free and bound phenolic compounds in both full-fat and defatted as compared to non hydrolyzed protein isolates (without enzymes), in addition new bands appeared as compared to control (incubation without enzymes).

Table 5.1 and Figure 5.1 show results from SDS-PAGE of hydrolyzed protein isolate from full-fat and defatted soybean before and after removal of free and bound phenolic compounds. The hydrolyzed protein isolate from defatted soybean before removal of phenolic compounds gave 5 major subunits with molecular weight 39.40, 27.5, 22.50, 19.50 and 17.41 KDa, while for hydrolyzed protein isolate after removal of free and bound phenolic compounds 3 major subunits with molecular weight of 27.55, 25.72 and 17.40 KDa were obtained.

The hydrolyzed protein isolate from full-fat soybean before and after removal of free and bound phenolic compounds gave 2 major subunits with molecular weight 27.60 and 17.50 KDa (Table 5.1 and Figure 5.2). Soybean glycinin and β-conglycinin were not detected in the SDS-PAGE in fermented soybean (Lijun *et al.*, 2003). Hrckova *et al.* (2002) reported that hydrolysis of glycinin and β-conglycinin produces peptide with molecular weight less than 20 KDa. β-conglycinin is easily hydrolyzed, while glycinin is highly resistant to enzymatic hydrolysis (Kim *et al.*, 1990; Marsman *et al.*, 1997; Schwingel and Bates, 1996). Polyphenolic compounds have ability to inhibit digestive enzymes; this can reduce the digestibility of food components such as proteins, carbohydrate and lipid (Long Staff *et al.*, 1993; Quesada *et al.*, 1996).

Figure 5.3 show the Native-PAGE and SDS-PAGE for hydrolyzed protein isolates from full-fat flaxseed before and after removal of free and bound phenolic compounds. Removal of free and bound phenolic compounds from protein isolates from full-fat and defatted flaxseed had no effect on the migration and molecular weight of protein subunits.

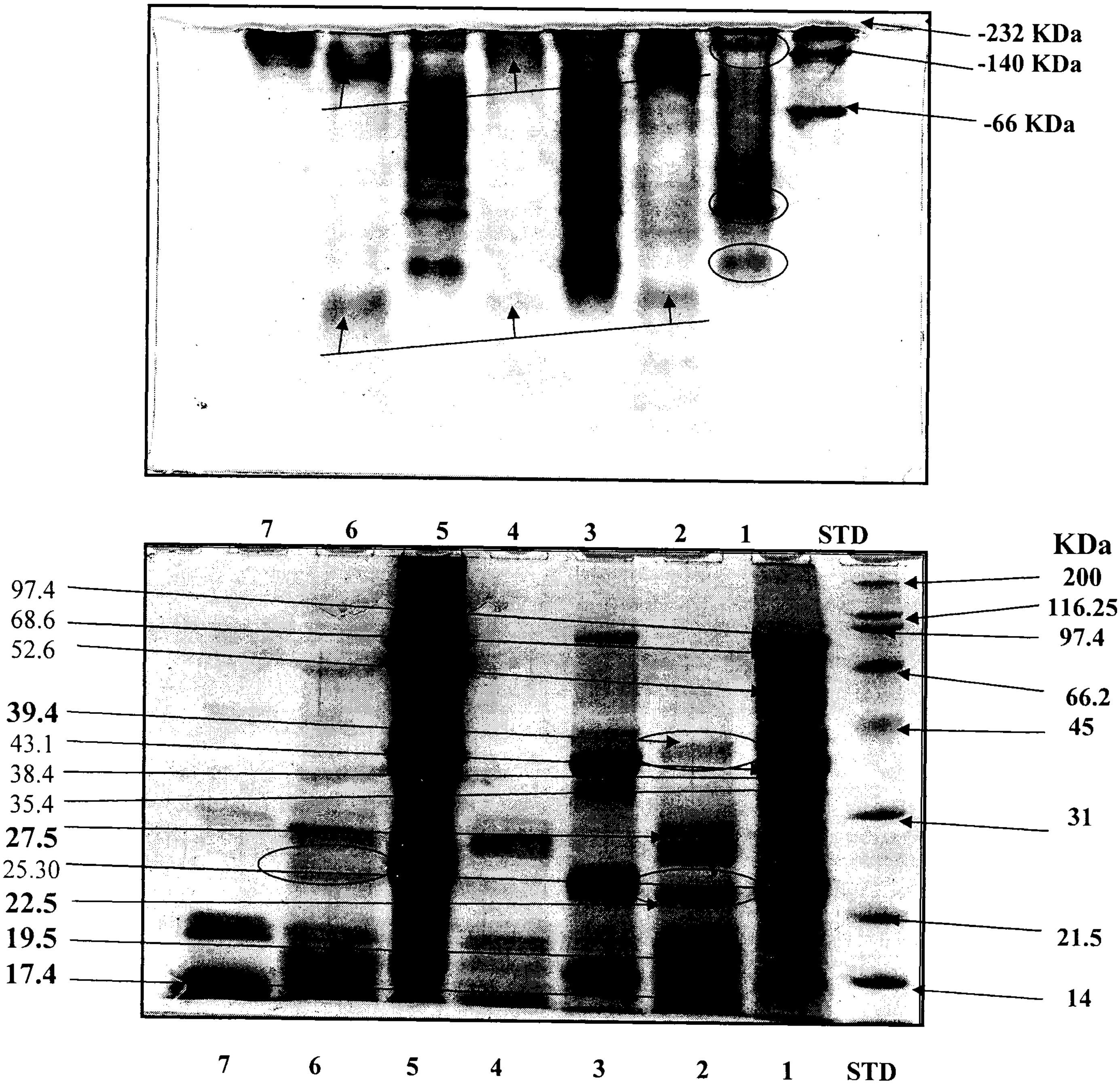


Figure 5.1: Native and SDS PAGE for protein isolate (I) from defatted (D) soybean (s); (1) Control DsI; (2) Hydrolyzed DsI; (3) Control DsI after removal of free phenolic compounds; (4) Hydrolyzed DsI after removal of free phenolic compounds; (5) Control DsI after removal of free and bound phenolic compounds; (6) Hydrolyzed DsI after removal of free and bound phenolic compounds; (7) Enzymes.

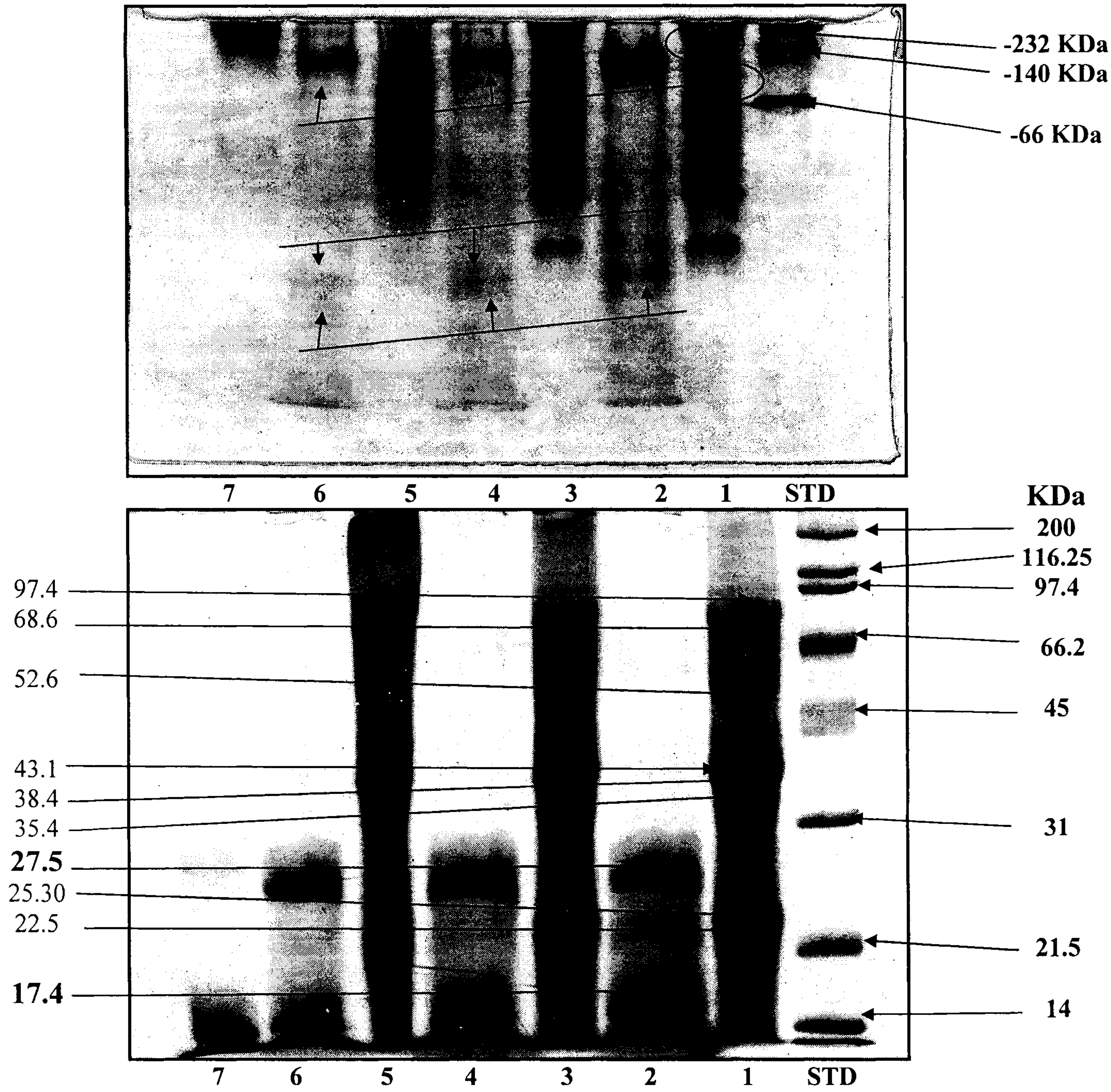


Figure 5.2: Native and SDS PAGE for protein isolate (I) from full-fat (F) soybean (s); (1) Control FsI; (2) Hydrolyzed FsI; (3) Control FsI after removal of free phenolic compounds; (4) Hydrolyzed FsI after removal of free phenolic compounds; (5) Control FsI after removal of free and bound phenolic compounds; (6) Hydrolyzed FsI after removal of free and bound phenolic compounds; (7) Enzymes.

Table 5.1: Effects of removal of free and bound phenolic compounds on molecular weight MW (KDa) from SDS-PAGE of subunits from hydrolyzed protein isolates (I) from full-fat (F) and defatted (D) soybean (s).

Observed MW[1] before removal of phenolic compounds				Observed MW after removal of free phenolic compounds		Observed MW after removal of free and bound phenolic compounds	
DsI	FsI	DsI	FsI	DsI	FsI	DsI	FsI
(withouht enzyme)		(with enzyme)		(with enzyme)		(with enzyme)	
ND	ND	39.4	ND	ND	ND	ND	ND
ND	ND	ND	ND	ND	ND	25.72	ND
ND	ND	27.5	27.60	27.5	27.55	27.55	27.60
22.5	22.5	22.5	ND	ND	ND	ND	ND
ND	ND	19.5	ND	ND	ND	ND	ND
ND	ND	17.41	17.50	17.41	17.49	17.40	17.40

ND: Not Detected

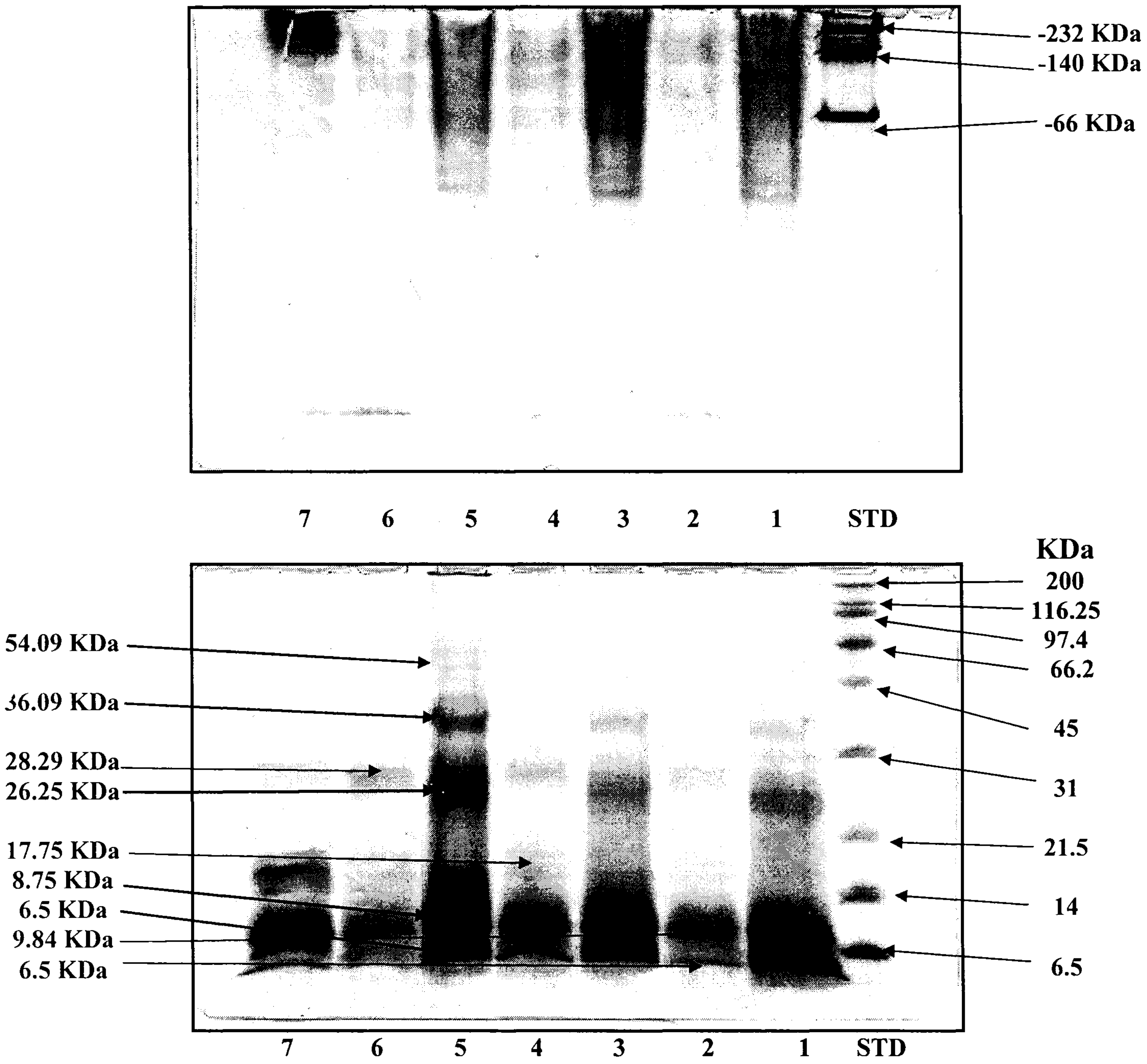


Figure 5.3: Native and SDS PAGE for protein isolate (I) from full-fat (F) flaxseed (f); (1) Control FfI; (2) Hydrolyzed FfI; (3) Control FfI after removal of free phenolic compounds; (4) Hydrolyzed FfI after removal of free phenolic compounds; (5) Control FfI after removal of free and bound phenolic compounds; (6) Hydrolyzed FfI after removal of free and bound phenolic compounds; (7) Enzymes. similar electropherogram was obtained for protein isolate from defatted.

5.3.2 RP-HPLC of Protein Hydrolysis

Figures 5.4, 5.5, 5.6, 5.7, 5.8 and 5.9 show the chromatograms obtained from RP-HPLC for hydrolyzed protein isolates before and after removal of free and bound phenolic compounds from full-fat and defatted soybean. For hydrolyzed protein isolate from full-fat and defatted soybean, the peptide profile obtained from RP-HPLC was similar to the hydrolyzed protein isolate after removal of free and bound phenolic compounds except for the absence of fraction Y (retention time 26.5) for hydrolyzed protein isolate before removal of free phenolic compounds. This suggests the hydrolysis of protein isolates from full-fat defatted soybean was affected by removal of free and bound phenolic compounds. RP-HPLC chromatogram for peptides obtained from hydrolyzed protein isolate after removal of free phenolic compounds from defatted soybean was slightly similar to hydrolyzed protein isolate from full-fat (Figure 5.8). Indicating that removal of lipid from the meal had no effect on the hydrolysis of the protein isolates from soybean.

For hydrolyzed protein isolates after removal of free and bound phenolic compounds, the peptide profile showed changes in the retention times of fraction Z, X, and Y from 20.9, 21.8, and 26.3 for hydrolyzed protein isolate from defatted soybean to 19.6, 22.5 and 27.2 for hydrolyzed protein isolate from full-fat soybean, respectively (Figure 5.9), indicating hydrolysis of the protein isolate after removal of free and bound phenolic compounds was affected by removal of lipid from the meal.

Figures 5.10 and 5.11 show RP-HPLC chromatograms of peptides obtained from hydrolyzed protein isolates before and after removal of phenolic compounds from full-fat and defatted flaxseed. The retention times of peptides obtained after hydrolyzed of protein isolate from both full-fat and defatted flaxseed before removal of phenolic compounds were similar to those of peptides obtained from protein isolate after removal of free and bound phenolic compounds.

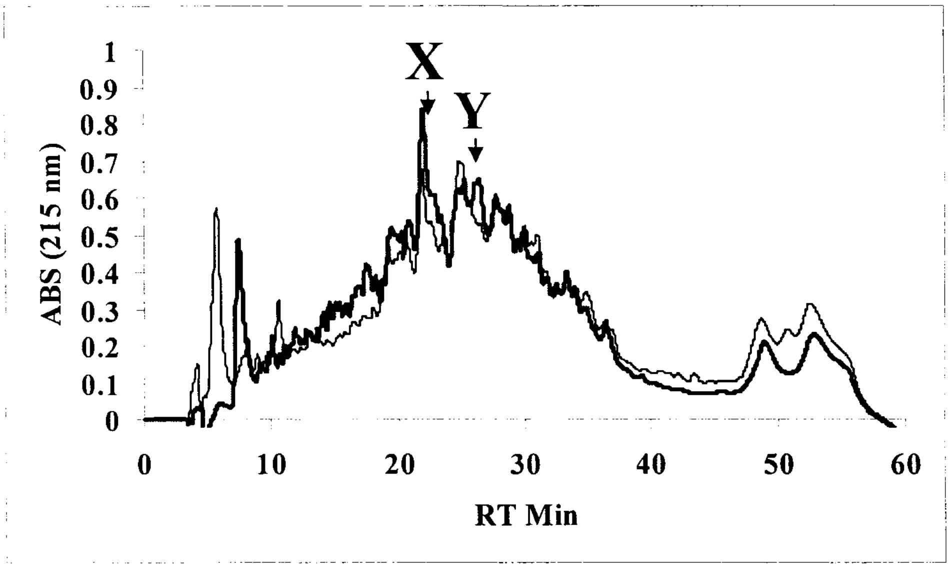


Figure 5.4: RP-HPLC chromatogram for soybean (s) protein isolate (I) from defatted (D); ▨ Hydrolyzed DsI; ■ Hydrolyzed DsI after removal of free phenolic compounds.

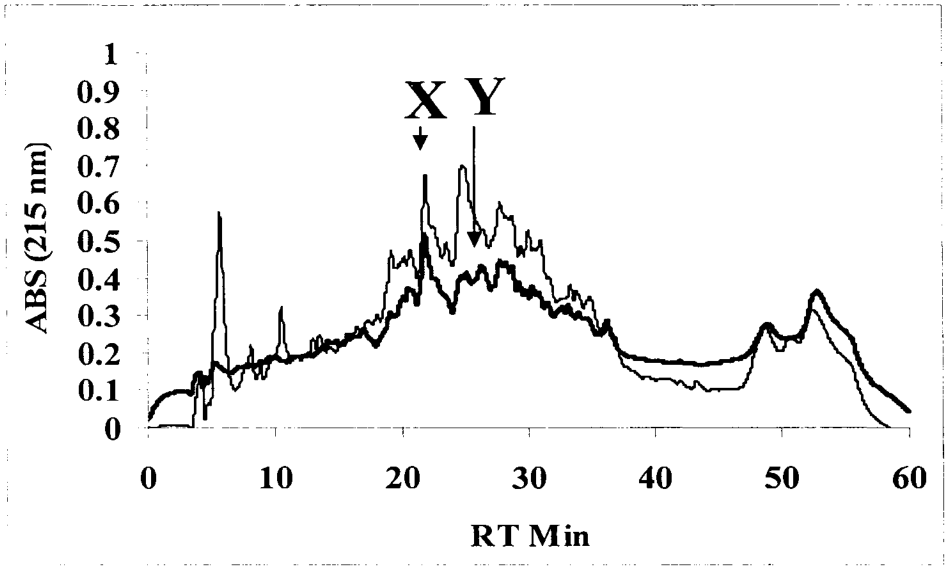


Figure 5.5: RP-HPLC chromatogram for soybean (s) protein isolate (I) from defatted (D); ▨ Hydrolyzed DsI; ■ Hydrolyzed DsI after removal of free and bound phenolic compounds.

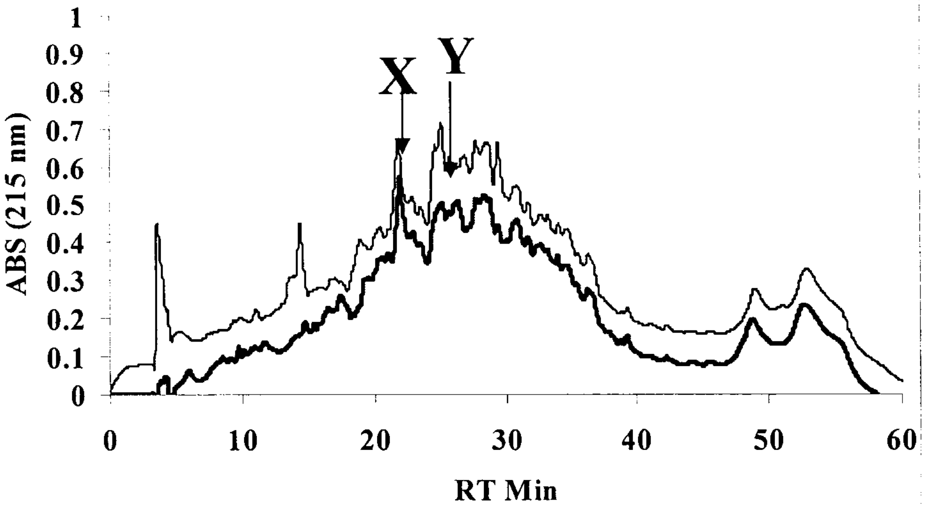


Figure 5.6: RP-HPLC chromatogram for soybean (s) protein isolate (I) from full-fat (F); ▨ Hydrolyzed FsI; ■ Hydrolyzed FsI after removal of free phenolic compounds.

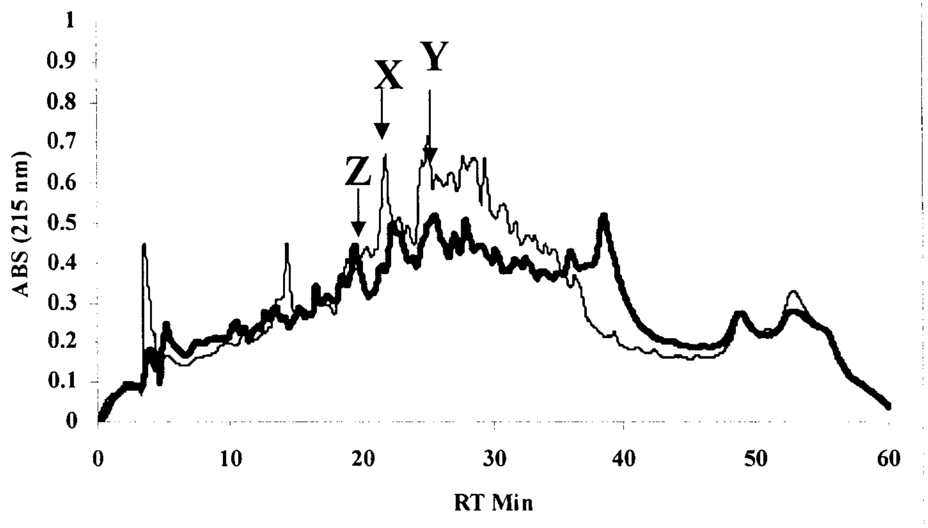


Figure 5.7: RP-HPLC chromatogram for soybean (s) protein isolate (I) from full-fat (F); ▨ Hydrolyzed FsI; ■ Hydrolyzed FsI after removal of free and bound phenolic compounds.

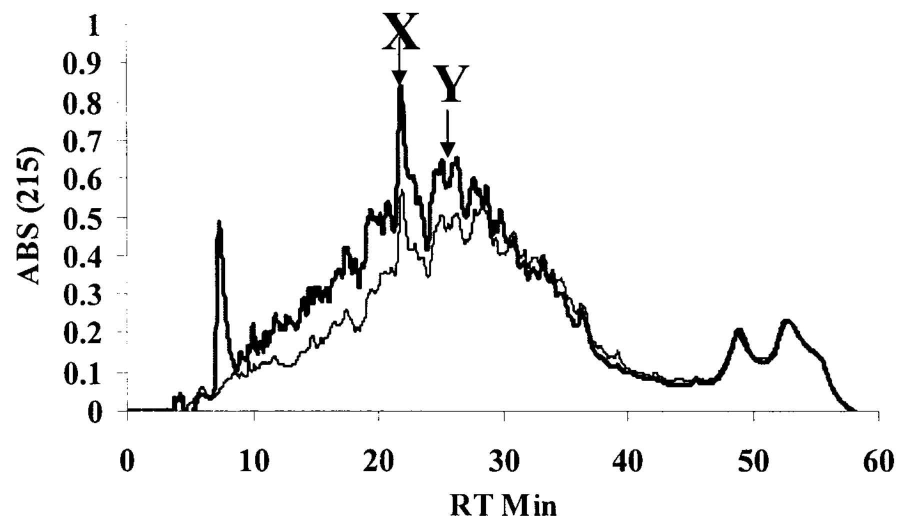


Figure 5.8: RP-HPLC chromatogram for soybean (s) protein isolate (I) after removal of free phenolic compounds ▩ Hydrolyzed FsI; ■ Hydrolyzed DsI.

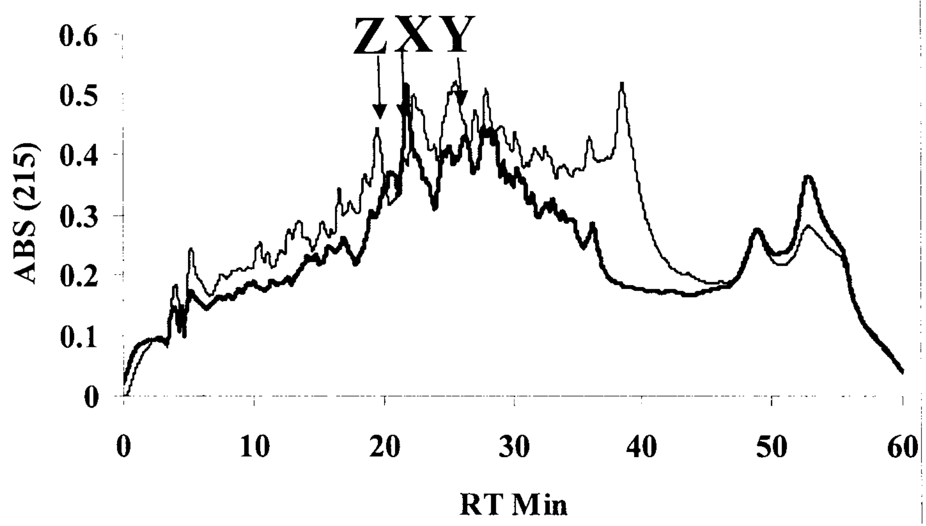


Figure 5.9: RP-HPLC chromatogram for soybean (s) protein isolate (I) after removal of free and bound phenolic compounds ▩ Hydrolyzed FsI; ■ Hydrolyzed DsI.

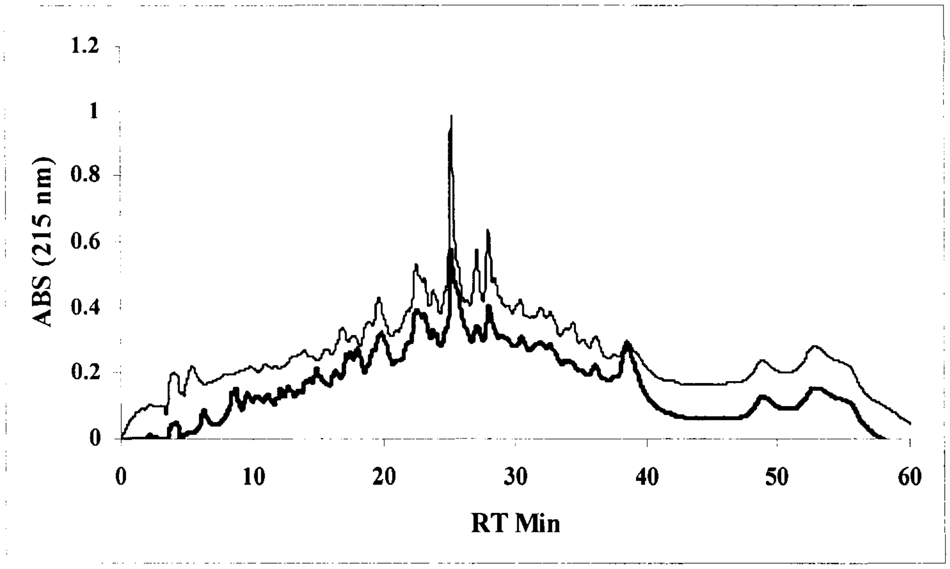


Figure 5.10: RP-HPLC chromatogram for flaxseed (f) protein isolate (I) from full-fat (F); ▪ Hydrolyzed FfI; ■ Hydrolyzed FfI after removal of free phenolic compounds; similar results were obtained for hydrolyzed of protein isolate from defatted.

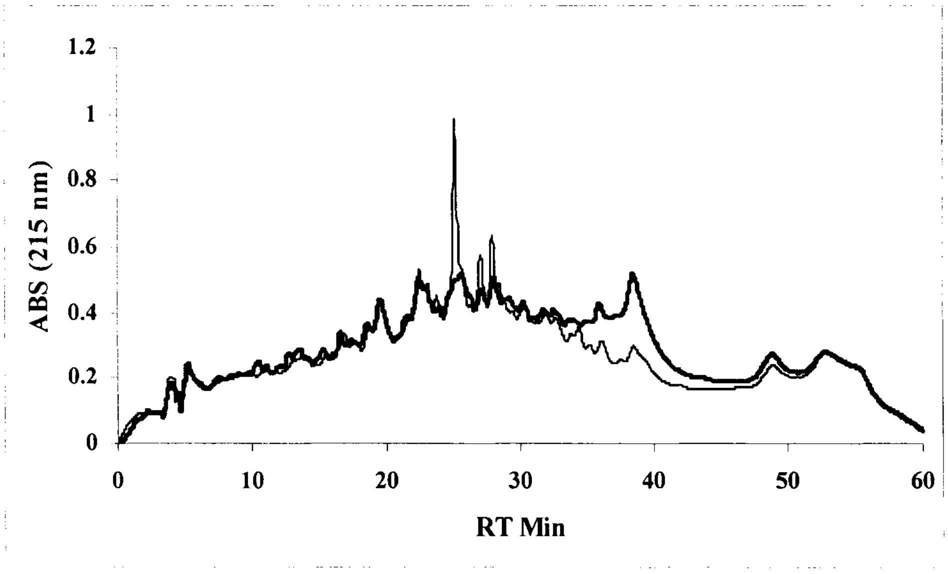


Figure 11: RP-HPLC chromatogram for flaxseed (f) protein isolate (I) from full-fat (F); ▪ Hydrolyzed FfI; ■ Hydrolyzed FfI after removal of free and bound phenolic compounds.; similar results were obtained for hydrolyzed of protein isolate from defatted flaxseed.

CHAPTER 6

EFFECT OF REMOVAL OF PHENOLIC COMPOUNDS ON PHYSICO-CHEMICAL PROPERTIES OF SOYBEAN AND FLAXSEED PROTEINS

6.1 Justification

This Chapter covers objective 4, which was aimed to investigate the effect of removal of free and bound phenolic compounds on physico-chemical properties of protein isolates obtained from full-fat and defatted flaxseed and soybean. Water holding capacity (WHC), differential scanning calorimetry (DSC) and rheometry were used to study thermal denaturation properties and gelling characteristics of the protein isolates.

6.2 Materials and Methods

6.2.1 Materials

Protein isolates from defatted and full-fat soybean and flaxseed were used; removal of free and bound phenolic compounds from the protein isolates is described in Sections 4.2.2, 4.2.3 and 4.2.4.

6.2.2 Differential Scanning Calorimetry (DSC)

Thermal denaturation temperature of protein isolates was carried out using the procedure described by Ahmed *et al.* (2006). Protein isolates were subjected to the thermal denaturation. Dispersions (5% NaCl, 20% W/V, pH 8) of all protein isolates were prepared. Thermal analysis was performed using a differential scanning calorimeter (DSC) (TA Q100, TA Instruments). The instrument was calibrated for temperature and heat flow using indium and sapphire standards. Nitrogen was used as purge gas at a flow rate of 50 ml/min. Hermetically sealed aluminum pans were used to avoid moisture loss during the analysis; a small sample of dispersion was sealed in the sample pan, weighed, cooled to −10°C in the DSC and held for 10 min for equilibration. The heating rate was 5°C/min over a range of 0°C to 150°C. A four-axis robotic device was used to automatically load the sample and reference pans to the DSC. The DSC data were analyzed translated into PRN format for Microsoft Excels manipulation and stored on disc. As reference, an empty aluminum pan used. All analysis were performed in duplicate.

6.2.3 Effect of Removal of Phenolic Compounds on Gelation Properties of Proteins

6.2.3.1 Preparation of Gels

The procedure for preparation of gels was carried out according to the method described by Boye *et al.* (1995). Aqueous dispersions (20% W/V) of protein isolates from were prepared in beakers using 5% NaCl and mixed well using magnetic stirrer. The pH was adjusted to pH 8 by addition of 0.1 NaOH solution. The beakers were covered with aluminum foil to prevent moisture loss. The protein dispersions were heated at 95°C for 30 min in constant temperature at water bath for gelation. The gels were cooled to 4°C for 24 h before water holding capacity and gelation studies.

6.2.3.2 Water Holding Capacity (WHC)

WHC of the gels prepared in Section 6.2.3.1 was determined for all protein isolates using a centrifugation technique (Kinsella, 1984). The gels were centrifuged at (10,000 X g, 15 min) and the supernatants that separated were measured. Water holding capacity was expressed as the water retained in the residue after centrifugation. All measurements were done in triplicate.

6.2.3.3 Rheological Properties of Protein Isolates

The rheological properties of the gels prepared in section 6.2.3.1 were determined for all protein isolates according to the method described by Ahmed *et al.* (2006) with modifications using a rheometer (AR 2000, TA Instruments, New Castle, DE, USA). A measured volume (approximately 2 ml) of sample was placed on the bottom plate of the rheometer. The experimental temperature was 22°C. The rheological properties of the protein gels were determined using a controlled stress rheometer, and the experimental dynamic rheological data were obtained directly from the TA Rheology Advantage Data Analysis software (V 5.1.42). The samples were subjected to oscillatory shear between two 60-mm parallel plates with a gap of 1000 μm. The AR 2000 was equipped with an efficient Peltier Temperature Control System, and the sample temperatures were precisely controlled and monitored. Dynamic rheological tests were performed within the linear viscoelastic region in the frequency (ω) range between 0.063 and 62.800 rad/s. Results were expressed as the storage modules (G′), loss modules (G″) and delta over time. All the rheological measurements were carried out in duplicate.

6.3 Results and Discussion

6.3.1 Effect of Removal of Phenolic Compounds on Denaturation Temperatures of Protein Isolates

Figures 6.1 and 6.2 and Table 6.1 show the effects of removal of phenolic compounds on the thermograms of DSC for soybean protein isolates from defatted and full-fat. Two denaturation temperatures were observed for protein isolate from defatted soybean (before removal of phenolic compounds) at 78.5°C and 122°C (Figure 6.1 and Table 6.1) corresponding to the denaturation temperatures of β-conglycinin and glycinin, respectively (Abdolgader, 2000). A slight decrease in denaturation temperature for glycinin to 120°C and 121.5°C after removal of free phenolic compounds and free and bound phenolic compounds, respectively. The denaturation peak for β-conglycinin was not observed after removal of free phenolic compounds. For protein isolates from full-fat soybean, the thermal denaturation temperature for glycinin was observed at 119°C before removal of phenolic compounds (Figure 6.1 and Table 6.1). The denaturation temperature for glycinin increased to 122.5°C after removal of free phenolic compounds from protein isolates, the denaturation peak for glycinin was not observed after removal of free and bound phenolic compounds. The results suggest that the removal of free phenolic compounds had only minor effect on the thermal denaturation for glycinin and β-conglycinin for protein isolate from soybean (Figure 6.2 and Table 6.1).

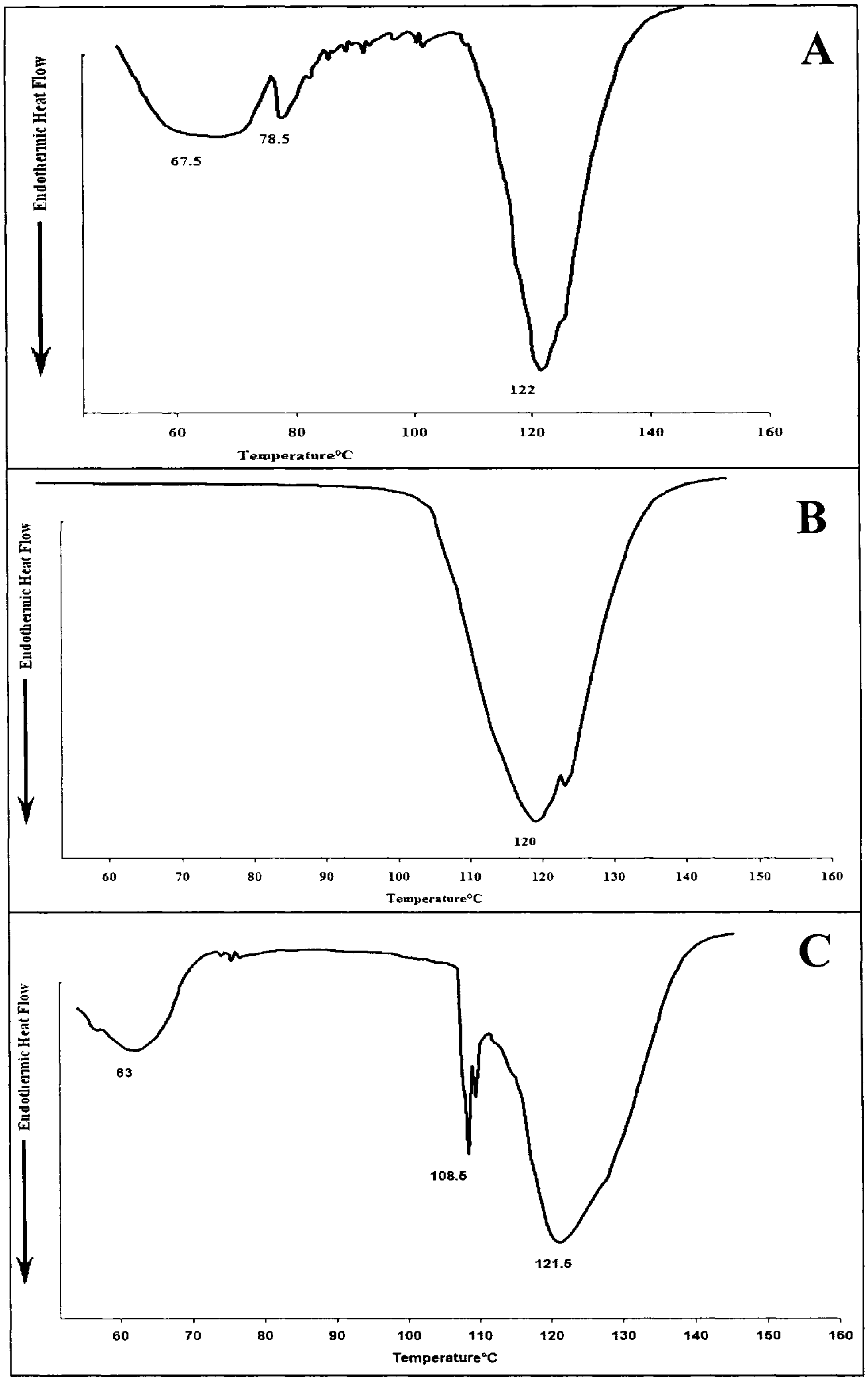


Figure 6.1: DSC thermograms of protein isolate (I) from defatted (D) soybean (s); (A) DsI; (B) DsI after removal of free phenolic compounds; (C) DsI after removal of free and bound phenolic compounds.

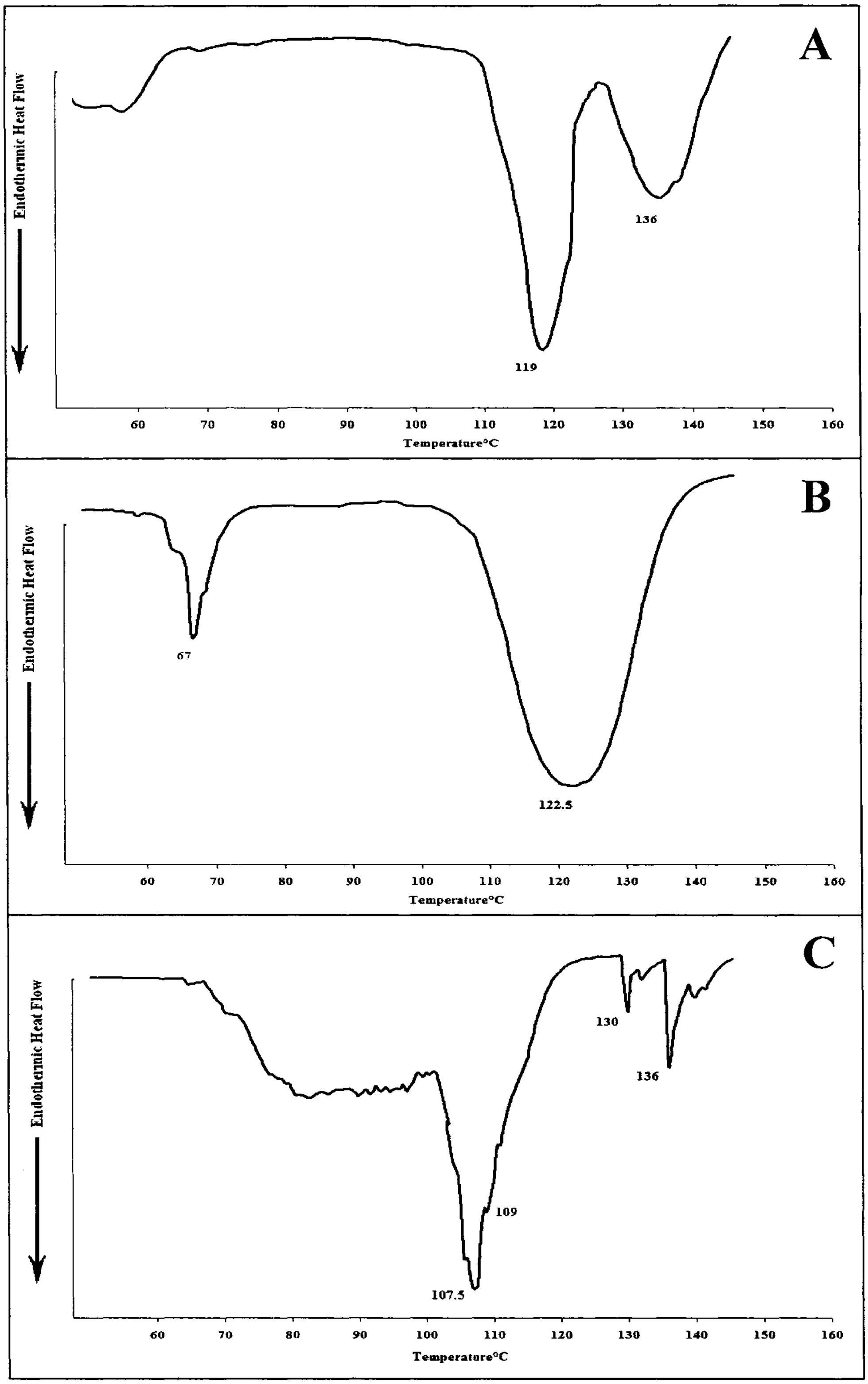


Figure 6.2: DSC thermograms of protein isolate (I) from full-fat (F) soybean (s); (A) FsI; (B) FsI after removal of free phenolic compounds; (C) FsI after removal of free and phenolic compounds.

Figures 6.3 and 6.4 and Table 6.1 show the DSC thermograms for protein isolates before and after removal of free and bound phenolic compounds from defatted and full-fat flaxseed. For protein isolate from defatted before removal of phenolic compounds; three denaturation temperatures were obtained at 121.6°C, 128°C and 140.8°C. Similar results were obtained for flaxseed protein isolates (90°C-120°C) (Li-Chan and Ma, 2002; Green *et al.,* 2005; Oomah *et al.,* 2006). Removal of free phenolic compounds from protein isolate for defatted flaxseed showed the denaturation temperatures of peaks at 128°C and 140°C were decreased to 126.5°C and 138.4°C, respectively. For protein isolates after removal of free and bound phenolic compounds; the denaturation temperature of the peak 128°C was decreased to 125.2°C (Figure 6.3 and Table 6.1). With protein isolate from full-fat flaxseed before removal of phenolic compounds, a thermal denaturation temperature peak was observed at 125.0°C. This denaturation temperature decreased to 123°C after removal of free and bound phenolic compounds. These results suggest that phenolic compounds from the protein isolate for full-fat and defatted flaxseed has only a minor reduction of the protein stability.

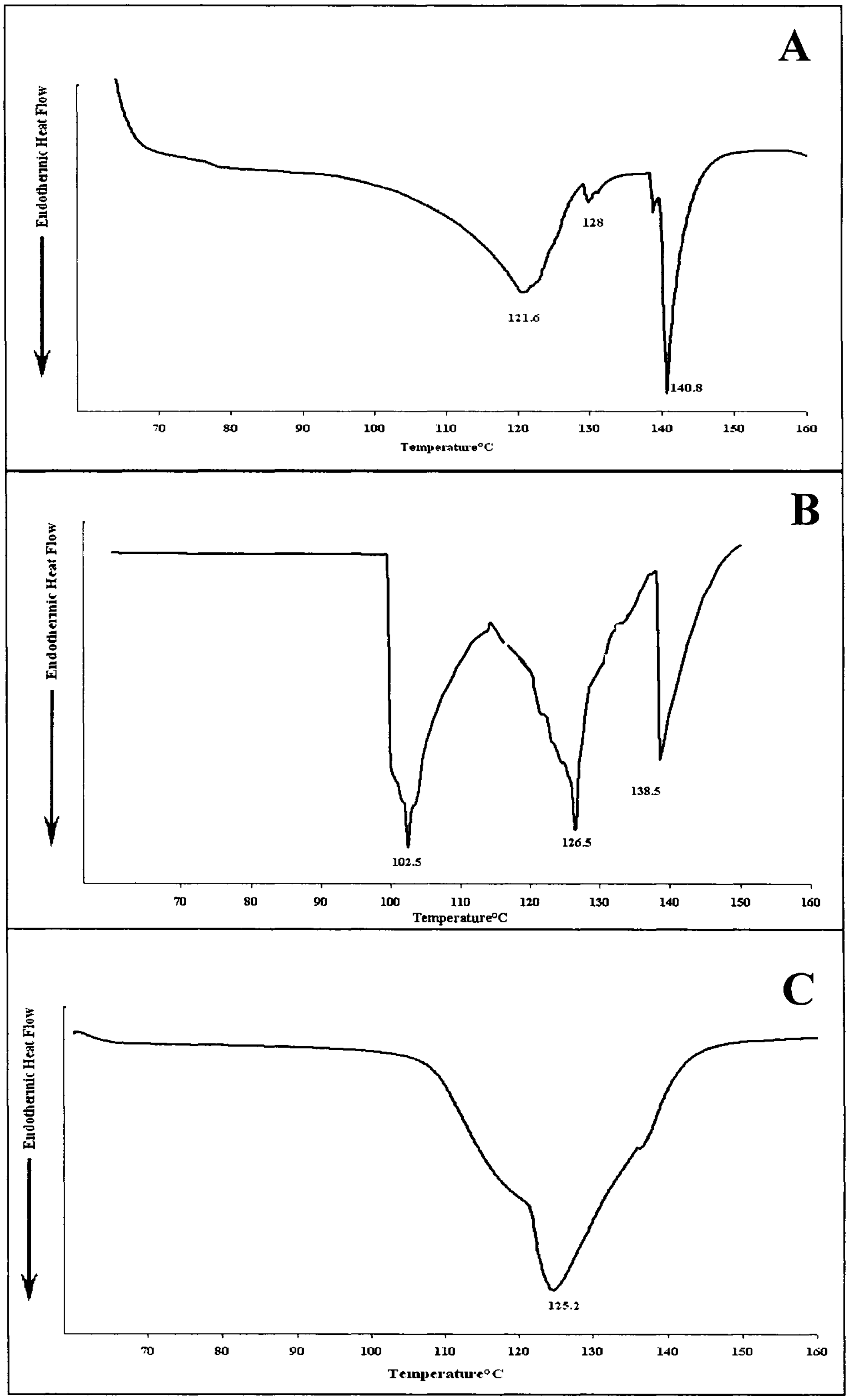


Figure 6.3: DSC thermograms of protein isolate (I) from defatted (D) flaxseed (f); (A) DfI; (B) DfI after removal of free phenolic compounds; (C) DfI after removal of free and bound phenolic compounds.

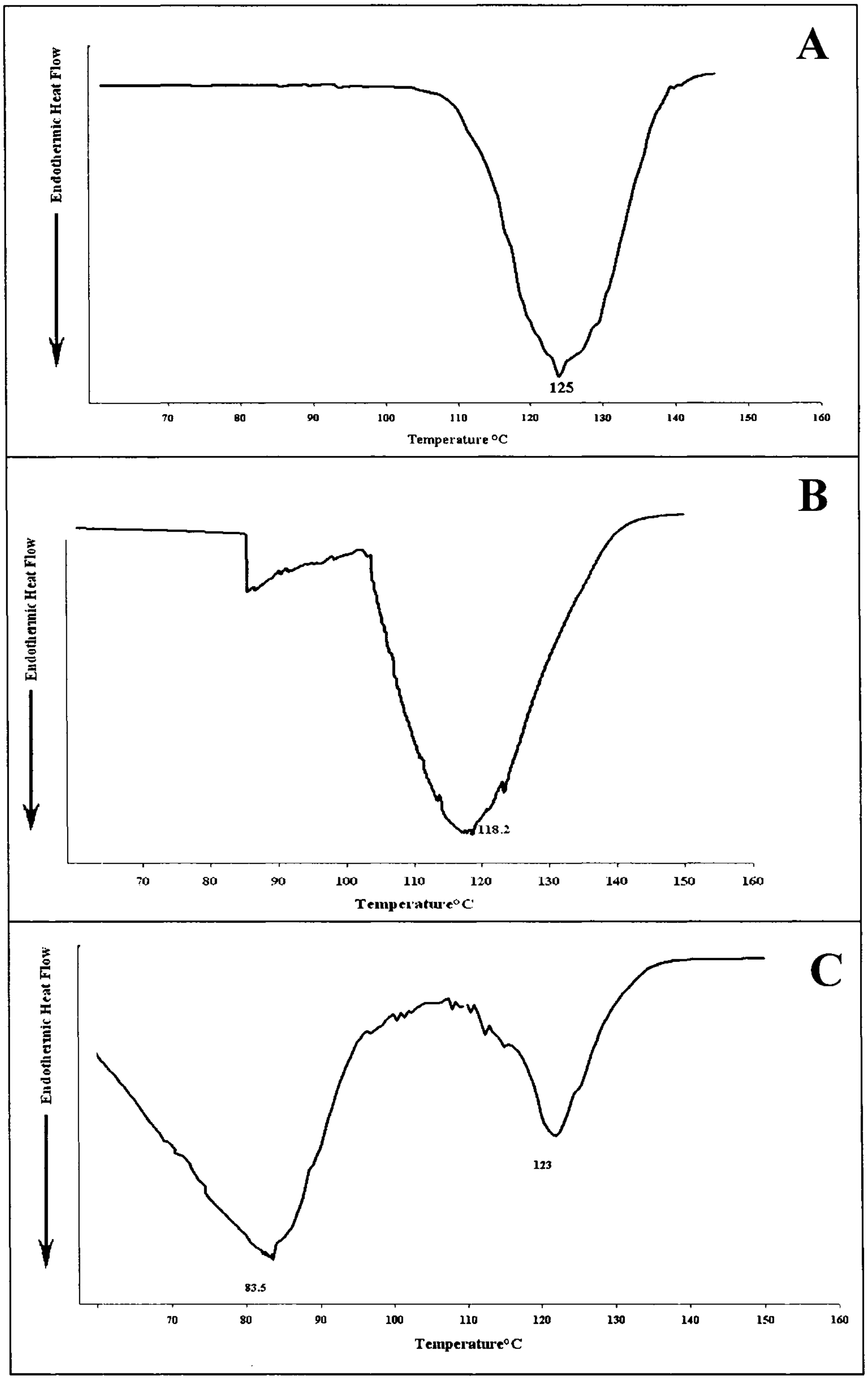


Figure 6.4: DSC thermograms of protein isolate (I) from full-fat (f) flaxseed (f); (A) FfI; (B) FfI after removal of free phenolic compounds; (C) FfI after removal of free and bound phenolic compounds.

Table 6.1: Denaturation temperature of protein isolate (I) from full-fat (F) and defatted (D) flaxseed (f) and soybean (s) before and after removal of free and free and bound phenolic compounds.

	Denaturation Temperature (°C)					
Protein Isolates	**Literature Value[a]**	**DsI**	**FsI**	**Literature Value[b]**	**DfI**	**FfI**
Before removal of phenolic compounds	72.8 91.1-115	78.5 122	60 119	90-120	121.6 128, 140.8	125
After removal of free phenolic compounds	NR	120	67 122.5	NR	102.5, 126.5 138.5	118.2
After removal of free and bound phenolic compounds	NR	63 121.5	107.5 136	NR	125.2	123

Results are means ± of two determinations.

NR: Not Reported

(a): Sheard *et al.* (1986); Damodaran (1988); Arrese *et al.* (1991); Wanger *et al.* (1996); Abdolgader (2000); Ahmed *et al.* (2006).

(b): Li-Chan and Ma (2002); Marcone *et al.* (1998); Oomah *et al.* (2006).

6.3.2 Effect of Removal of Phenolic Compounds on Water Holding Capacity (WHC) of Protein Isolates

Table 6.2 and Figure 6.5 show the effect of removal of free and bound phenolic compounds on WHC of protein gels from full-fat and defatted flaxseed and soybean. Generally, gels obtained from protein isolates for defatted meals showed higher WHC compared to gels obtained from protein isolates for full-fat except for protein gel after removal of free phenolic compounds from flaxseed and protein gel after removal of free and bound phenolic compounds from soybean. Difference in WHC can be related to the protein content of protein isolates (Liu, 1999). Gels prepared after removal of free and bound phenolic compounds from protein isolates from full-fat meals showed higher WHC (86% for flaxseed and 99% for soybean) compared to gel prepared before removal of phenolic compounds (59% for flaxseed and 74% for soybean) and for gels after removal of free phenolic compounds (70% for flaxseed and 77% for soybean) (Table 6.1 and Figure 6.5). Kinsella (1984) reported that the WHC is affected by protein composition especially the number of polar groups, protein conformation and surface hydrophobicity. Gel prepared from protein isolate from defatted flaxseed before removal of phenolic compounds, showed higher WHC 98%, followed by gels prepared from protein isolate after removal of free and bound phenolic compounds (93%), and protein isolate after removal of free phenolic compounds (63%). The gel prepared from protein isolates of defatted soybean after removal of free and bound phenolic compounds gave minimum WHC 88%. The gel prepared from protein isolate for defatted soybean before removal of phenolic compounds had similar WHC (98%) to gel obtained after removal of free phenolic compounds. WHC for protein isolates from soybean was reported to be 80%-98% (Abdolgader, 2000; Alzagtat, 2003).

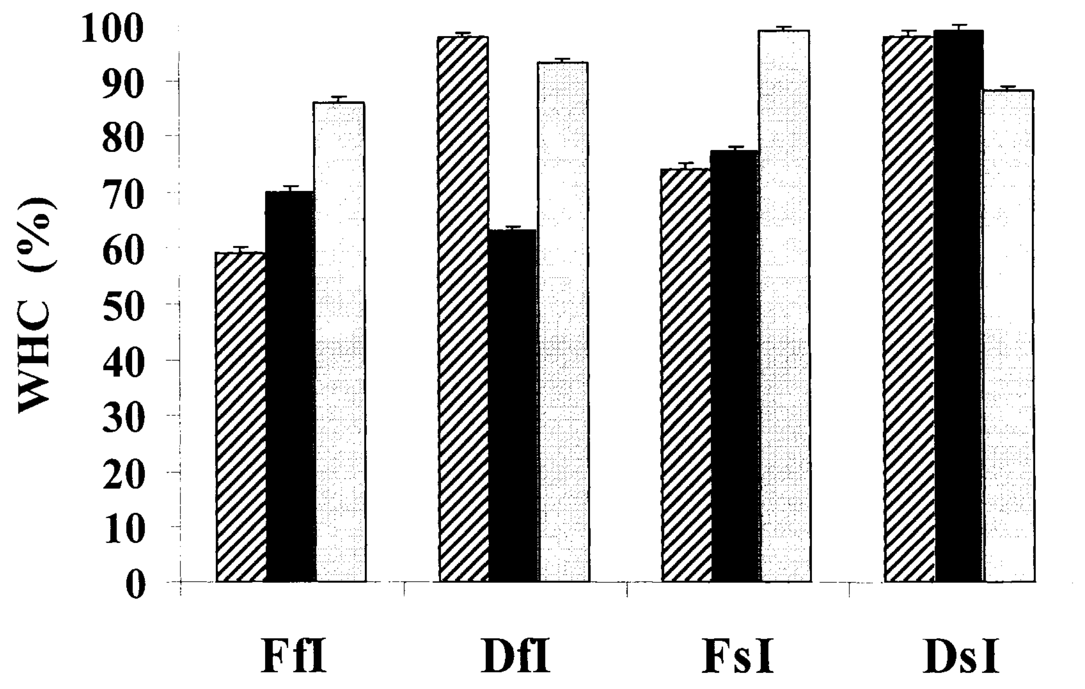


Figure 6.5: WHC for protein isolate (I) from defatted (D) and full fat (F) of soybean (s) and flaxseed (f); ▨ Protein isolates before removal of phenolic compounds; ■ after removal of free phenolic compounds; □ after removal of free and bound phenolic compounds.

Table 6.2: Determination of water holding capacity (WHC%) for protein isolates (I) from full-fat (F) and defatted (D) soybean (s) and flaxseed (f).

Protein Isolates	FfI*	DfI*	FsI*	DsI*
Before removal of phenolic compounds	59.0±1.0	98.0±0.6	74.0±1.0	98.0±1.0
After removal of free phenolic compounds	70.0±1.0	63.0±0.6	77.0±1.0	99.0±1.0
After removal of free and bound phenolic compounds	86.0±1.0	93.0±1.0	99.0±0.6	88.0±0.6

Results are means ± standard deviations of three determinations.

6.3.3 Effect of Removal of Phenolic Compounds on Gelation Properties of Protein Isolates

Figures 6.6, 6.7, 6.8 and 6.9 showed the effect of removal of free and bound phenolic compounds from protein gels from defatted and full-fat flaxseed on the storage modules, loss modulus and tan (delta). G' results for all samples was greater than G" values, suggesting the gel network was more elastic and less viscous. Gels from the protein isolate (defatted meal) after removal of free and bound phenolic compounds showed lowest storage moduli (G'), loss moduli (G"), followed by the (G') and (G") from protein isolate after removal of free phenolic compounds from (defatted meals); while the (G') and (G") of gels prepared from protein isolate before removal of phenolic compounds showed the highest gelation properties for protein isolates from defatted meals (Figure 6.6 and Figure 6.7). This suggests that the presence of free and bound phenolic compounds in protein isolates from defatted meal enhanced gelation properties.

For full-fat protein isolates from soybean and flaxseed, removal of free phenolic compounds result in (G') and (G") showing higher gelation properties compared with protein isolates before removal of phenolic compounds. (G') and (G") values for the gel of protein isolates obtained after removal of free and bound phenolic compounds indicated the lowest gelation properties (Figure 6.8 and Figure 6.9). Suggesting the presence of free phenolic in protein isolates enhanced the viscoelastic properties for protein gels from full-fat meals. Generally, the gels for protein isolates from full-fat and defatted meals after removal of free phenolic compounds showed the enhanced gelation properties as compared to gelation properties of protein isolates after removal of free and bound phenolic compounds (Figure 6.10 and 6.11).

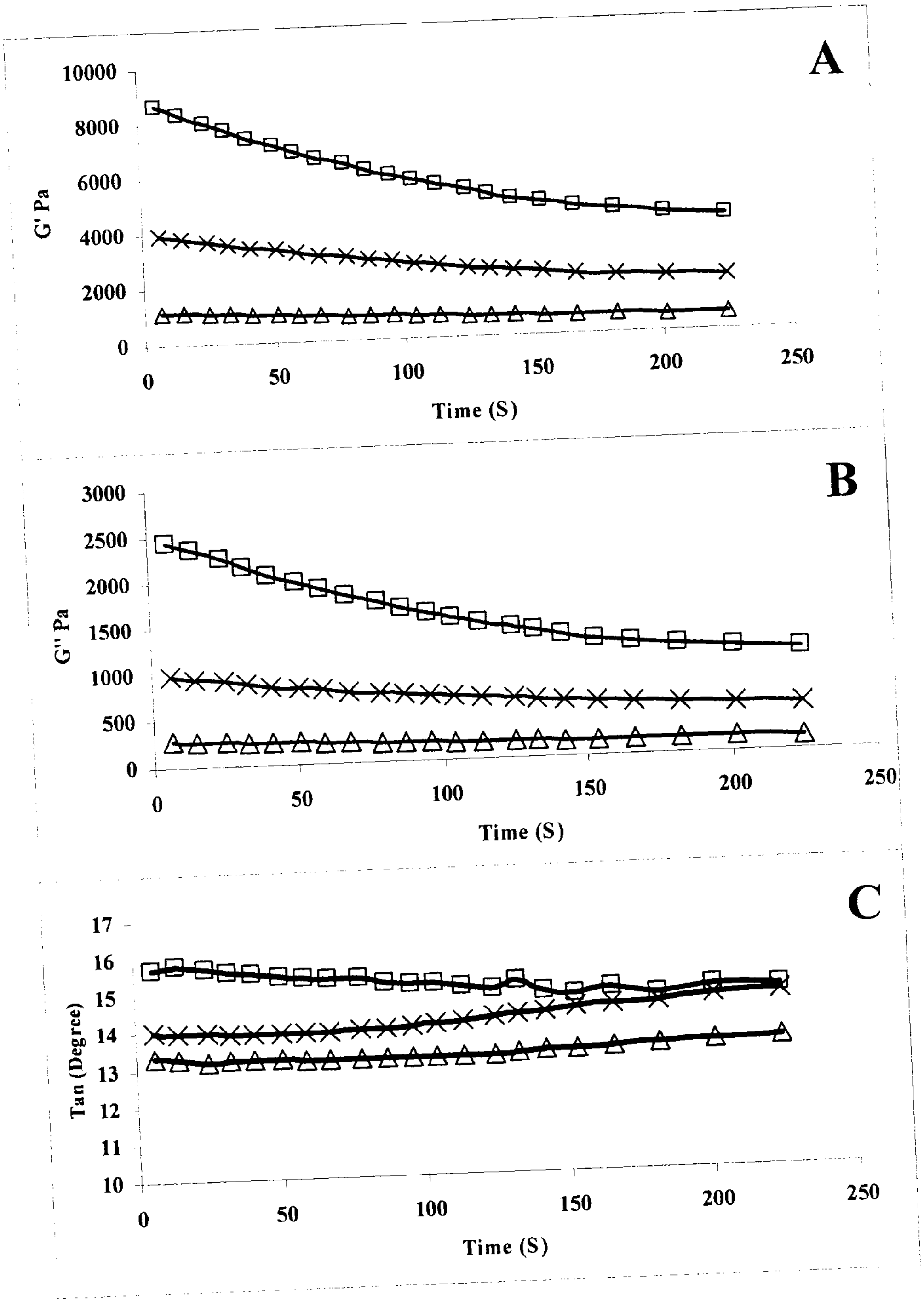


Figure 6.6: Changes in dynamic viscoelastic modules with time, (A) changes in storage moduli (G'); (B) changes in loss moduli (G''); (C) change in delta (tan) for soybean (s) protein isolate (I) from defatted (D);—□— DsI;—×— DsI after removal of free phenolic compounds;—△— DsI after removal of both free and bound phenolic compounds.

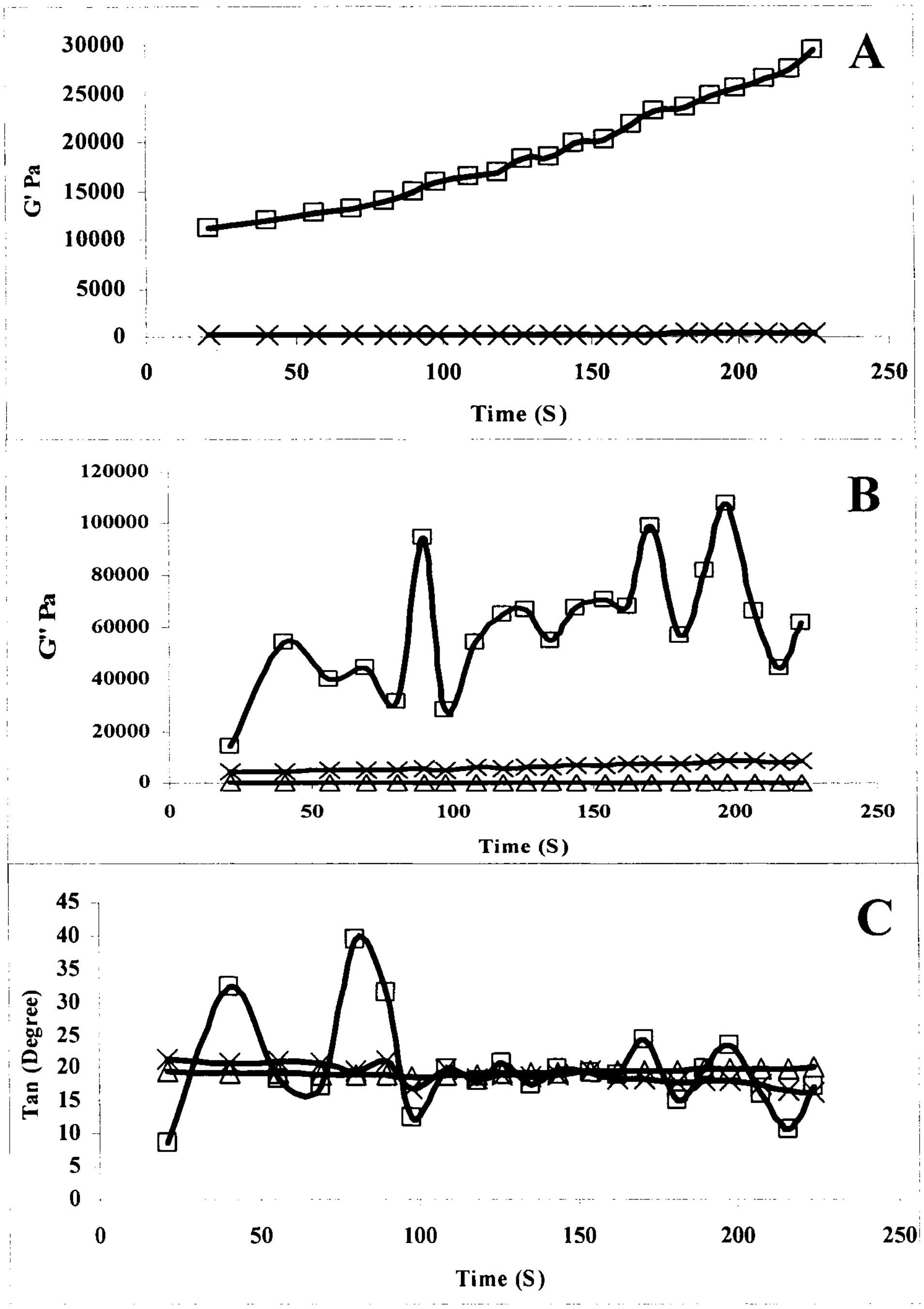


Figure 6.7: Changes in dynamic viscoelastic modules with time, (A) changes in storage moduli (G'); (B) changes in loss moduli (G"); (C) change in delta (tan) for flaxseed (f) protein isolate (I) from defatted (D);—⊟— DfI;—×— DfI after removal of free phenolic compounds;—△—DfI after removal of free and bound phenolic compounds.

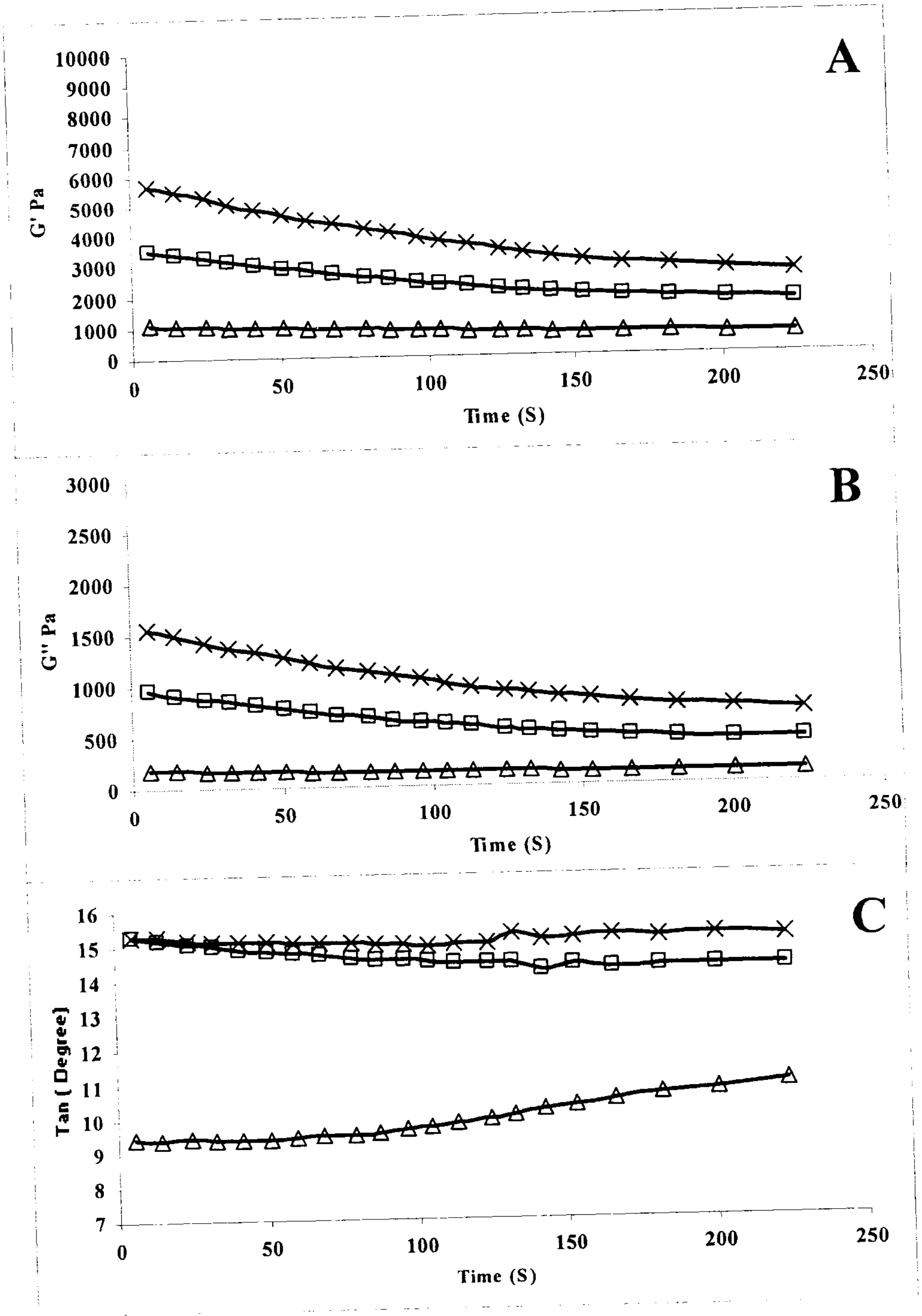


Figure 6.8: Changes in dynamic viscoelastic modules with time, (A) changes in storage moduli (G'); (B) changes in loss moduli (G''); (C) change in delta (tan) for soybean (s) protein isolate (I) from full-fat (F); —⊟— FsI; —✕— FsI after removal of free phenolic compounds; —△— FsI after removal of both free and bound phenolic compounds.

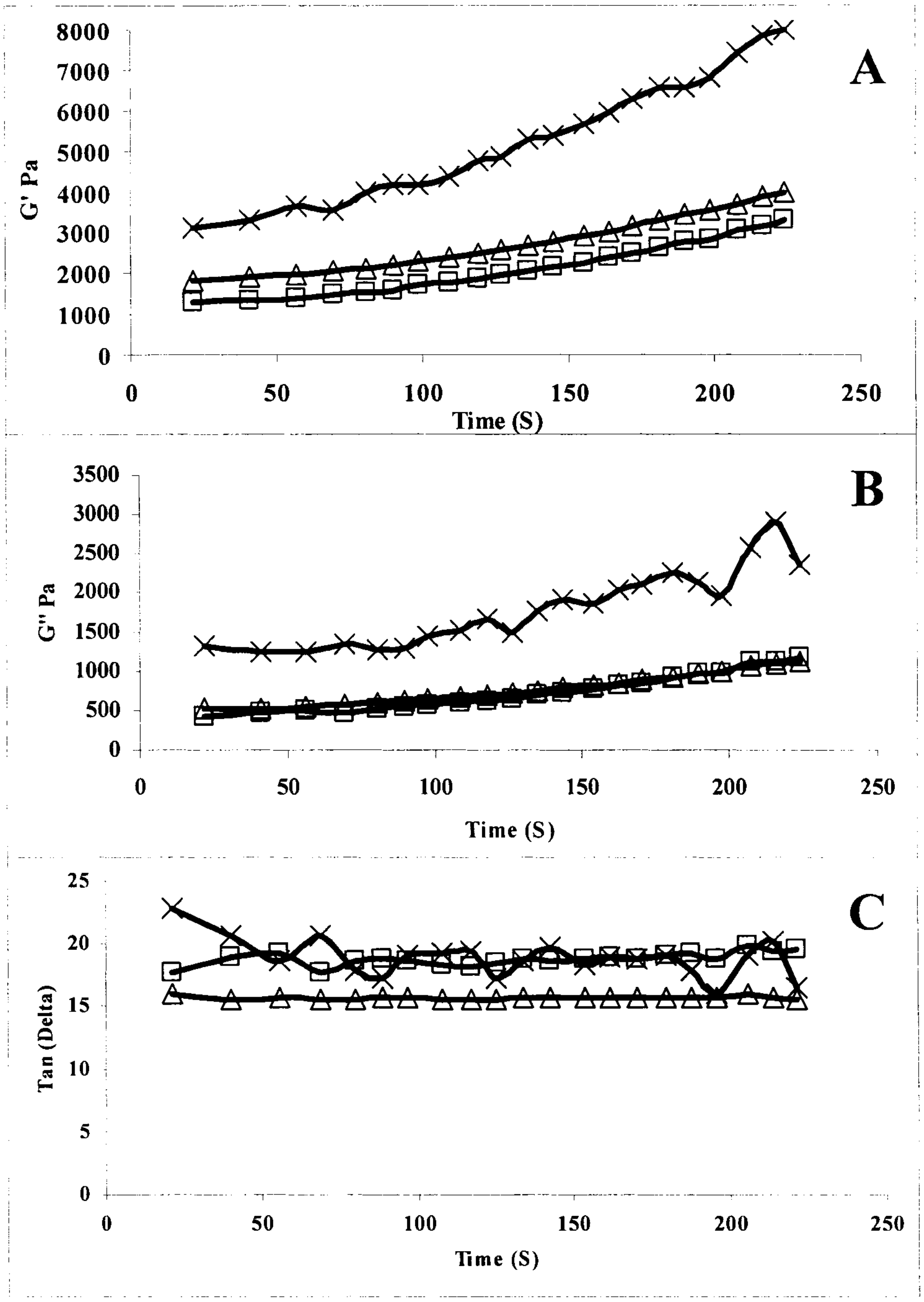


Figure 6.9: Changes in dynamic viscoelastic modules with time, (A) changes in storage moduli (G'); (B) changes in loss moduli (G"); (C) change in delta (tan) for flaxseed (f) protein isolate (I) from full-fat (F); —□— FfI; —×— FfI after removal of free phenolic compounds; —△— FfI after removal of free and bound phenolic compounds.

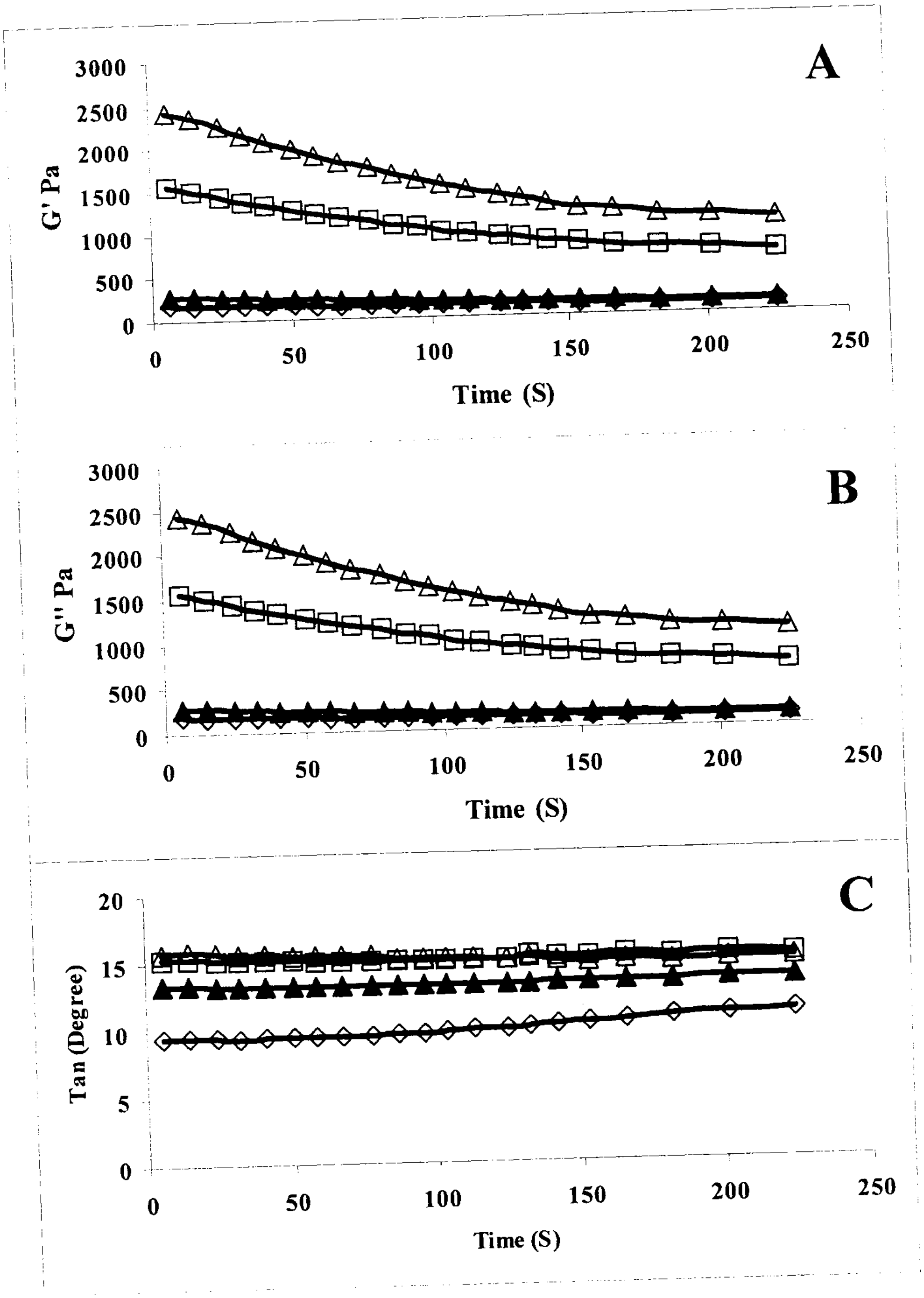


Figure 6.10: Changes in dynamic viscoelastic modules with time, (A) changes in storage moduli (G'); (B) changes in loss moduli (G''); (C) change in delta (tan) for soybean (s) protein isolate (I) from full-fat (F) and defatted (D); —△— DsI after removal of free phenolic compounds; —▲— DsI after removal of free and bound phenolic compounds; —□— FsI after removal of free phenolic compounds; —◇— FsI after removal of free and bound phenolic compounds.

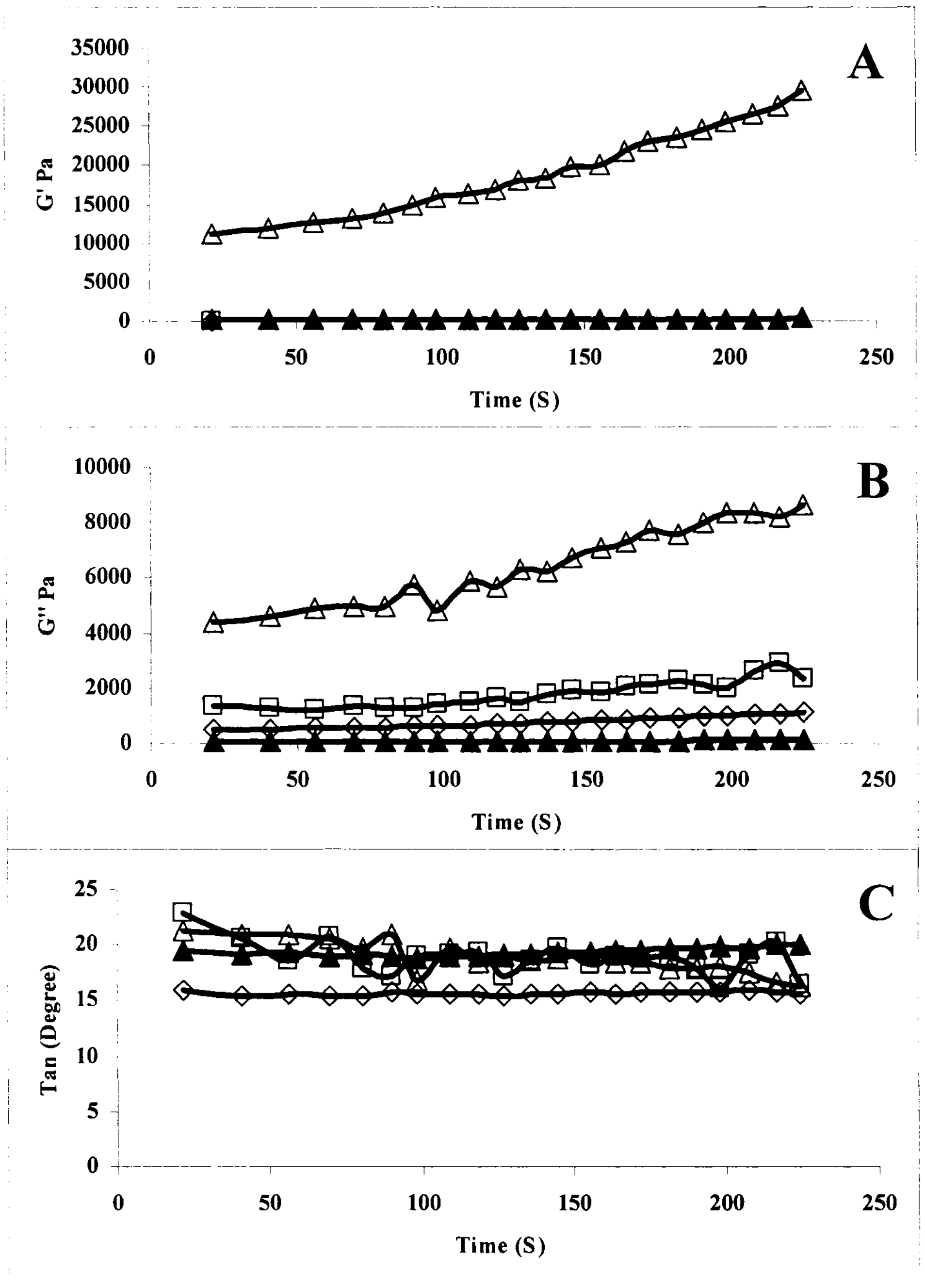


Figure 6.11: Changes in dynamic viscoelastic modules with time, (A) changes in storage moduli (G'); (B) changes in loss moduli (G''); (C) change in delta (tan) for flaxseed (f) protein isolate (I) from full-fat and defatted (D);—△— DfI after removal of free phenolic compounds;—▲— DfI after removal of free and bound phenolic compounds; —□— FfI after removal of free phenolic compounds; —◇—FfI after removal of free and bound phenolic compounds.

GENERAL CONCLUSIONS

This study was investigated the phenolic compounds in oil-bearing plants and the effects of these compounds on molecular characteristics, biological properties and physico-chemical properties of protein isolates from full-fat and defatted soybean and flaxseed. The total free and total bound phenolic compounds for flaxseed meals and their protein isolates were higher when compared to soybean meals and their protein isolates. The results obtained from RP-HPLC profiles for protein isolates from soybean and flaxseed indicated some differences in the phenolic compounds designated as free and bound phenolic compounds. Sinapic acid was present in the protein isolate for defatted flaxseed but not in the protein isolate for full-fat meal. Generally, the antioxidant activity of full-fat meals and their protein isolates for both free and bound phenolic compounds higher than defatted. The bound phenolic compounds in protein isolates from both flaxseed and soybean showed antioxidant activity except the bound phenolic compounds in protein isolate from defatted soybean which showed no antioxidant activity.

Removal of free and bound phenolic compounds had only minor effects on the molecular characteristics of protein isolates from full-fat and defatted soybeans. Native-PAGE, SDS-PAGE and RP-HPLC for the peptides profiles from both full-fat and defatted soybean protein isolates revealed the removal of free and bound phenolic compounds had minor effects on the biological properties of protein isolates. Protein isolates from defatted soybean after removal of free and bound phenolic compounds were showed a decrease in thermal stability of glycinin, decrease WHC and gave less viscous and more elastic gels as compared to protein isolate before removal of phenolic compounds. For protein isolate from full-fat soybean, the results showed the removal of free phenolic compounds increase thermal stability of glycinin with increase WHC and produce more viscous and less elastic gels as compared to protein isolate before and after removal of bound phenolic compounds. Removal of free and bound phenolic compounds from protein isolates from full-fat and defatted flaxseed decrease thermal stability, water holding capacity (WHC) and viscoelastic properties as compared to protein isolate before removal of phenolic compounds and after removal of free phenolic compounds.

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